Research Article

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PCR ASSAY BASED MOLECULAR CHARACTERIZATION OF *JUSTICIA BETONICA* LINN., *THOTTEA SILIQUOSA* LAM., *MEMECYLON EDULE* ROXB., *PAJANELIA LONGIFOLIA* (WILLD) K. SCHUM AND *BAUHINIA ACCUMINATA* LINN

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ABSTRACT

Characterization of plants with DNA markers is an ideal approach for identification of medicinal plant species as they provide objectively analyzable data for accurate and fast identification. There are many medicinal plants described in ancient literatures that have not been exploited for their medicinal values. A documented treasure of traditional knowledge of Kerala's Ayurvedic and folklore practices is the 'Hortus Malabaricus'. Description of various useful medicinal plants and their use as single drug can be seen in this ethnomedicinal compendium. The plants *Justicia betonica* Linn., *Thottea siliquosa* Lam., *Memecylon edule* Roxb., *Pajanelia longifolia* (Willd) K. Schum.) and *Bauhinia accuminata* Linn described in Hortus Malabaricus are considered to possess immense therapeutic potentials. As the Ayurveda industry as well as practitioners are facing severe scarcity of required medicinal plants, search for establishing the claimed pharmacologic values of hitherto unexplored plants is to be promoted. For this purpose, their proper identification and authentication is needed as a first step of scientific experiments. The molecular characterization including DNA isolation and sequencing provides fast and accurate identification of medicinal plant species. In this project, the molecular characterization of *Justicia betonica*, *Thottea siliquosa*, *Memecylon edule*, *Pajanelia longifolia* and *Bauhinia accuminata*, including DNA isolation and quantification, NCBI-BLAST analysis and DNA sequencing was done. The sequence analysis of *Thottea siliquosa*, *Memecylon edule*, *Bauhinia accuminata* and *Justicia betonica* by BLASTN (NCBI) is revealed 99 to 100 % nucleotide sequence homology with different database sequences enabling species level confirmation.

Keywords: *Justicia betonica* Linn., *Thottea siliquosa* Lam., *Memecylon edule* Roxb., *Pajanelia longifolia* (Willd) K. Schum.), *Bauhinia accuminata* Linn.

INTRODUCTION

Medicinal plants, the cardinal factors in Ayurveda, have been used worldwide for centuries to maintain health and for disease management¹. WHO says a vast majority of world population relies on the plant-based systems of medicine for their primary healthcare needs. However, adulteration and use of spurious materials as substitutes have become a major concern for users and industry for reasons of safety and efficacy, underlining the need for proper authentication of medicinal plants. Quality control has always been a key issue in the development and authentication of herbal medicines. Characterization of plants with DNA markers is an ideal approach for identification of medicinal plant species². DNA markers offer several advantages over conventional phenotypic markers, as they provide data that can be analyzed objectively and also assures accurate and fast scientific identification of the plant.³

There are many medicinal plants described in ancient literatures that have been unexploited for their medicinal values. One such documented treasure of traditional knowledge of Kerala's

Ayurvedic and folklore practices is Hortus Malabaricus. *Justicia betonica* Linn., *Thottea siliquosa* Lam., *Memecylon edule* Roxb., *Pajanelia longifolia* (Willd) K. Schum.) and *Bauhinia accuminata* Linn., described in Hortus as plants having immense potentials are among the least explored ones.

Bauhinia acuminata Linn. locally known as Velutha Mandaram⁴, is an erect deciduous flowering shrub found almost throughout India⁵. This ethnomedicinal plant belonging to family Fabaceae has been in use for oedema, fever, inflammation, rheumatism, skin diseases and ulcer⁶. Anti-microbial, anti-oxidant, anti-inflammatory and anti-depressant activities of this plant have been already investigated by scientists^{7,8}.

Locally known as Venkurinhi⁹, *Justicia betonica* Linn., belonging to family Acanthaceae, is distributed widely in India and Srilanka^{10,11}. It is used as a remedy for various fevers, stomach pain, diarrhoea, inflammation and swelling^{12,13}. The plant possess many analgesic, anti-inflammatory, anti-malarial, anti-microbial properties and were tested against many pathogenic bacteria^{12,13}.

Memecylon edule Roxb, locally known as Kasavu¹⁴, belonging to family Melastomaceae is a plant widely used by the ethnic communities in Kerala for healing ulcers¹⁵. Moreover, a decoction prepared from its leaves is used internally for gonorrhoea, stomach pain and fever symptoms. Previous research reports exist on the anti-inflammatory, anti-microbial and antioxidant potentials of this species.¹⁶⁻¹⁸

Thottea siliquosa Lam. distributed mainly throughout South Western Ghats of India¹⁹; belongs to family Aristolochaceae. This wonderful plant is locally known by name Alpam²⁰ or Kotasari and is being considered by Ayurvedic physicians and traditional healers of Kerala as an antitoxic agent mainly for poisonous bites^{21,22}. It is also used as a remedy for conditions like diarrhea, dysentery, cholera, inflammations, ulcers²³ etc.

Pajanelia longifolia (Willd) K. Schum is a sparingly branched tree with large, ornamental flowers belonging to family Bignoniaceae²⁴ and is locally known as Payyani.²⁵ It has anti-oxidant, antibacterial²⁶ anti-inflammatory, hepatoprotective anticancer etc activities and is used in many ailments like headache, arthritis, swellings, ulcers²⁷ etc.

Ayurveda industry and practitioners are facing severe scarcity of herbal drugs due to depletion of natural habitats. It is need of the hour to conduct research on unexplored plants with medicinal value claimed by traditional and folklore healers. The primary step is their proper identification and authentication for further scientific validations. DNA markers are reliable for information as the genetic composition is unique for each species and is not affected by age, physiological conditions, as well as environmental factors. DNA characterization provides fast scientific identification of the plant hence saves much time for further scientific studies².

Objective

Molecular characterization with DNA isolation and quantification, NCBI-BLAST analysis and DNA sequencing of *Thottea siliquosa* Lam, *Memecylon edule* Roxb., *Pajanelia longifolia* (Willd) K. Schum, *Bauhinia accuminata* Linn. and *Justicia betonica* Linn.

MATERIAL AND METHODS

The young leaves of the plants *Justicia betonica* Linn., *Thottea siliquosa* Lam., *Memecylon edule* Roxb., *Pajanelia longifolia* (Willd) K. Schum and *Bauhinia accuminata* Linn. were collected from its natural habitat. Chemicals and other accessories required for the assay were purchased from authorized dealers. The study was carried out at the Department of Computational Biology and Bioinformatics, University of Kerala, Karyavattom, Thiruvananthapuram.

Molecular characterization

Molecular characterization consists of

1. Genomic DNA isolation
2. Quantification Qubit Fluorometer
3. UV spectroscopy
4. Agarose gel electrophoresis
5. Polymerase Chain Reaction

Genomic DNA isolation

Isolation of DNA, RNA and proteins is essential for all molecular biology investigations. The DNA isolation from plant tissues is

very difficult due the presence of various secondary plant products. Here Genomic DNA was isolated according to the method described by Doyle and Doyle (1990) with slight modification.²⁹

Young leaf tissues (100 mg) were ground well with a motor and pestle using 700 mL of 2% CTAB extraction buffer [20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB, 0.4% β -mercaptoethanol added just before use]. The solution was transferred to a sterile 1.5 ml Eppendorf tube, added 10 μ L of Proteinase K and incubated at 65°C for 45 min. Following incubation, the tubes were centrifuged at 12,000 rpm for 10 minutes in room temperature. The supernatant was carefully transferred to a fresh tube and added 500 μ L of chloroform-isoamylalcohol (24:1) and mixed gently for 1 min. The samples were centrifuged at 12,000 rpm for 10 minutes in room temperature (this procedure was repeated twice). The aqueous phase obtained was then transferred to a fresh 1.5 ml Eppendorf tube and added 100 μ L of 3M sodium acetate (pH 3.2), followed by addition of 700 μ L of ice-cold isopropanol. The tubes were gently mixed by inversion and incubated at -20°C for 30 min. Following incubation, the tubes were centrifuged at 12,000 rpm for 10 min at 4 °C. The DNA adhered to the bottom of the tube was retained and the supernatant was carefully discarded. The DNA pellet was then washed with 700 mL of 70% ethanol to eliminate salt residues adhered to the DNA and set to dry approximately for 30 min with the tubes inverted over a filter paper, at room temperature. The pellet was then re-suspended in 100 μ L TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) plus 5 μ L ribonuclease (RNase 10 mg mL⁻¹) in each tube; this solution was incubated at 37°C for 30 min and after that stored at -20° C.²⁸

DNA quantification and electrophoresis

Quantification of DNA is the process to determine the average concentration of DNA. Agarose gel electrophoresis is used to separate DNA fragments for extraction and purification. DNA quantification was done with the aid of Qubit Fluorometer and also by 0.7 % Agarose gel electrophoresis followed by ethidium bromide visualization with a high range DNA ladder (100 bp to 10 Kb). In addition, the absorbance ratios at A_{260/280} were determined with the aid of spectrophotometer to evaluate the quality of the isolated DNA.^{30,31}

Qubit Fluorometer procedure

The required number of 0.5-mL tubes for standards and samples was set up. The Qubit® dsDNA HS Assay requires 2 standards. Prepared Qubit® working solution by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer. The final volume in each tube must be 200 μ L. Each standard tube requires 190 μ L of Qubit® working solution, and each sample tube requires anywhere from 180–199 μ L; prepared sufficient Qubit® working solution to accommodate all standards and samples. Added 190 μ L of Qubit® working solution to each of the tubes used for standards; added 10 μ L of each Qubit® standard to the appropriate tube, then mixed by vortexing 2–3 seconds. Added Qubit® working solution to individual assay tubes so that the final volume in each tube after adding sample is 200 μ L. Sample can be anywhere from 1–20 μ L; added a corresponding volume of Qubit® working solution to each assay tube: anywhere from 180–199 μ L; added each sample to the assay tubes containing the correct volume of Qubit® working solution; then mixed by vortexing 2–3 seconds. The final volume in each tube was 200 μ L; allowed all tubes to incubate at room temperature for 2 minutes. Finally, readings were taken in Fluorometer

Agarose gel electrophoresis

Dissolved 0.7 g Agarose in 100 ml of 1X TAE buffer (Prepared from 50 X stock) with the aid of microwave oven; allowed the solution to cool up to ~50° C. Added 0.5 µl ethidium bromide (stock:10 mg ml⁻¹), mixed slowly and poured onto gel casting tray for solidification; placed appropriate combs for well formation. The gel was kept undisturbed for 45 min. After 45 min placed the gel in electrophoresis tank containing tank buffer (1X TAE) and comb was removed. Mixed the samples with 1 X gel loading dye and loaded onto the wells. The samples were then run at 60 V for half an hour. Following gel run, the gel was placed on a gel documentation system for image capturing.

Conventional PCR using standardized program

The maximum and optimum annealing temperature per primer pair was empirically investigated by gradient PCR with a range of annealing temperatures starting from 45 to 60 °C for RbCL primers and 50 to 60 °C for MatK primer pair. Further PCR amplification was performed at the standardized annealing temperature. The PCR cycling profile for each primer set are tabulated and shown (Table 1). Each 25 µl PCR reaction consisted of 50 ng of template DNA, 0.2 mM deoxyribonucleotide triphosphate (dNTP), 0.2 µM of each primer, 1.5 mM MgCl₂, 1 X Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.01 % gelatin) and 1U of Taq DNA polymerase (Bangalore GeNei, India). Amplifications were performed in thermal cycler. The amplified products were resolved on a 1.2 % Agarose gel containing 0.5 µl (10 mg ethidium bromide ml⁻¹) ethidium bromide and photograph was scanned through Gel Doc System.

PCR product purification, sequencing and NCBI-BLAST analysis

The single amplified PCR product of each primer set was purified using PCR purification kit (Jena Bioscience, Merck) as per manufacturer's instruction. The purified PCR products were sent for sequencing (RGC, TVM). The nucleotide sequences obtained were processed to low-quality reads using the Bio Edit Sequence Alignment Program. The nucleotide sequences were then analyzed with NCBI nucleotide BLAST tool. The sequences were then submitted to Genbank database.

RESULT AND DISCUSSION

DNA of good quantity and quality was successfully obtained using the modified protocol. DNA quantity analysis using Qubit Fluorometer ranged from 78.6 to 100 µg/ml of DNA indicating the presence of higher DNA concentration. Results from 0.7 % Agarose gel electrophoresis showed sharp and clear bands of DNA (Figure 1) that are devoid of impurities. The absorbance at A_{260/280} (Spectrophotometer analysis) ranged from 1.79 to 1.88, suggesting that the DNA fraction was pure (Table 2), Gradient PCR assays using RbCL (Figure 2.a, 2.b, 2.c) and MatK primers enabled identification of apt annealing temperatures. Amplification of genomic DNA of *Memecylon edule*, *Pajanelia longifolia*, *Bauhinia accuminata* and *Justicia betonica* using RbCL primers yielded single products of ~ 600 bp (Figure 3) and MatK yielded ~1000 bp product (Figure 4) for *Thottea siliquosa*. The sequence analysis of *Thottea siliquosa*, *Memecylon edule*, *Bauhinia accuminata* and *Justicia betonica* by BLASTN (NCBI) revealed 99 to 100 % nucleotide sequence homology with different database sequences of *Thottea siliquosa* (Figure 5), *Memecylon edule* (Figure 6), *Bauhinia accuminata* (Figure 7) and *Justicia betonica* (Figure 8), thereby enabling species level confirmation. Sequence submissions for longifolia species of genus *Pajanelia* or *Bignonia* was not available in the database.

BLAST of analysis of *Pajanelia longifolia* sequence revealed 80 to 85 % sequence similarity with plant species belonging to genus *Bignonia* (Figure 9). Thus, molecular characterization of *Pajanelia longifolia* was possible only to the genus level. The sequences obtained were finally submitted to NCBI GenBank database.

Sequences submitted to NCBI GenBank

Bauhinia acuminata (Rbcl)

GTTTTTTTGGGGTCCAAAGCTTGGTGTAAAGATTATA
AATTAACCTATTATACTCCTAACTATGAAACCAAAGAT
ACTGATATCTTGGCAGCATTCGAGTAACCTCTCAACC
TGGAGTTCCTCCTGAAGAAGCAGGTGCCGCGGTAGCT
GCTGAATCTTCTACTGGTACATGGACAACCTGTGTGGAC
CGATGGGCTTACCAGTCTTGATCGTTACAAAGGACGAT
GCTACCACATCGAGCCCGTTGCTGGAGAAGAAAATCA
ATATATTGCCTATGTAGCTTATCCCCTAGACCTTTTGA
AGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGG
GTAATGTATTTGGATTCAAAGCCCTGCGCGCTCTACGT
CTGGAGGATTTGCGAATCCCTACTGCTTATATTAAC
TTTCCAAGGTCCGCTCACGGCATCCAAGTTGAGAGAG
ATAAATTGAACAAGTATGGTCGTCCTTATGGGATGT
ACTATTAACCTAAATTGGGGTTATCCGCTAAGAATTA
CGGTAGAGCAGTTTATGAATGTCTTCGCGGTGGACTTG
ATTTTACCA

Memecylon edule (Rbcl)

GTGTTGGATTCAAAGCTGGTGTAAAGATTATAAGCTG
ACTTATTATACTCCTGACTATGAAACCAAAGATACTGA
TATCTTGGCAGCATTCGAGTAACCTCTCAACCCGGAG
TTCCGCCGGAAGAAGCAGGGGTGCGGTAGCTGCTGA
ATCTTCTACTGGTACATGGACAACCTGTGTGGACTGATG
GGCTTACCAGCCTTGATCGTTATAAAGGAAGATGCTAC
CACATCGAGCCTGTTGCTGGAGAAGAAAATCAATATA
TATGTTATGTAGCTTACCCTTTAGACCTTTTGAAGAA
GGTCTGTTACTAATATGTTTACTTCCATTGTAGGGAA
TGTATTTGGCTTCAAAGCCCTTCGAGCTCTACGTCTGG
AGGATTTGCGAATCCCTACTTCCCTATATTAACCTTTC
CAAGGACCGCCTCATGGCATCCAAGTTGAGAGAGATA
AATTGAACAAGTATGGCCGTCCCCTGTTAGGATGTACT
ATTAACCGAAATTAGGTTTATCCGCTAAGAATTACGG
TAGAGCGGTTTATGAATGTCTTCGTGGTGGACTTGATT
TTACGAA

Justicia betonica (Rbcl)

CGGGGGTTTGGGGATTAAGCGGGTGTAAAGAGTACA
AATTGACTTATTATACTCCTGAATACGAAACCAAAGAT
ACTGATATCTTGGCAGCATTCGAGTAACCTCTCAACC
CGGAGTTCGCGCTGAAGAAGCAGGAGCCGCGGTAGCT
GCGGAATCTTCCACCGGTACATGGACAACCGTGTGGA
CCGATGGACTTACCAGTCTTGATCGTTACAAAGGGCGA
TGCTACAACATCGAGCCCGTCTTGGTGAACAGATCA
ATATATCTGTTATGTAGCTTACCCTTTAGACCTTTTGA
AGAAGGTTCTGTTACCAACATGTTTACTTCCATTGTGG
GAAATGTGTTTGGATTCAAAGCCTTGCGTGTCTACGT
CTGGAAGATCTTCAATCCCTACTGCTTATATTAACAA
TTTCCAAGGTCCGCTCATGGGATCCAAGTTGAGAGAG
ATAAGTTGAACAAGTATGGGCGTCCCCTGCTGGGATGT
ACTATTAACCAAATTTGGGGTTATCCGCTAAAACTA
TGGTAGAGCGTGTATGAATGTCTTCGCGGTGGATTGG
ATTTTACCA

Thottea siliquosa (MatK)

TAATTTACAATCTATTTCATTCAATATTTCCCTTTTTTCGA
GGACACATTATCACATTTAAATCATGTATTAGATATCC
TAATACCCTACTCCATCCATCCTGAAATCTTGCTTCAA
ACCCTTCACTGCTGGATACAAGATGCTCCTTCTTTGCA
TTTATTGCGATTCTTTCTCCATGAGTATCGTAGTTTCAT
TAGTCCAAAGAAATTCATTTCTTTTTTCTCAAAGGAGA
ATCAAAGGTTATTATTGTTTCGTATATAATTCTCATGTAT
ATGAATGCGAATCCATATTTGTGTTTCTCCGTAAACAA
TCCTCTCTTTTACGATCAACGTCCTCTGGAACCTTTCT
GAGCGAACATATTTCTATGGAATAAAGAACATTTTGT
AGTAGTGCTTTGTAATGATTTTAAAGAACCTGTGTT
TGTTCAGGATCCTTTTGCACATTATGTCAGATACCGA
GGAAAAGTCATTTTGACTTCAAAAAGGACTTATCTTAT
GATGAAGAAATGGAATCTCACCTGTAAATTTATGGC
AGTGTCAATTTTACTTGTGGTCACAACCGGGCAGAAATC
CATATAAACCGATTAGACAATTATTCCTTCTATTTAT
GGGCTATCTTTCAAGTCGACGAATAAACCTTGGGTGG
TAAGGAGTGAAATGCTAGAAAGTTCATTTATAATAGA

TACTTCCATTAAGAGATTTCGAGGCCATAGTCCCAATTA
TTTCTCTGATTAGTTGCTTGGTTAAAGCCAAATTTTGTA
ATGTATCGGGGCGTCCCATTAGTAAGCCGGCATGGGC
CGATTCTGCTGATTCTGATATTATCGATAGATTCTGGGC
GTATATGCAGAAATCTTTCTCATTATTACAGTGGATCT
TCAAAAAAACATAGTTTGCATCGAATCAAATATATACT
TCGACTTTCGTGT

Pajanelia longifolia (Rbel)

CCATAACTGCTCTCCATAGTTTTTACGAGCTAATCCCA
ATTTAGGTTTAAATAGTACATCCCAACAGGGGACGACC
ATACTTGTTCATTTATCTCTCTCAACTTGGATCCCATG
AGCGGGGCTTGGAAAGTTTTAATATAAGCAGGAGGG
ATTTCGAGATCTTCCAGACGTAGAGCACGCAGGGCTTT
GAATCCAAATACATTTCTTACAATGGAAGTAAACATGT
TAGTAACAGAACCTTCTTCAAAAAGGTCTAAAGGGTA
AGCTACATAACAGATAAATTGATCAGC

Table 1: Universal Primer Name and Sequence and PCR Programme

Universal Primer Name and Sequence	PCR programme
RbelF : 5' ATGTCACCACAAACAGAGACTAAAGC 3' RbelR : 5' GTAAAATCAAGTCCACCRG 3'	94 °C for 2 min 94 °C for 30 s 56 °C for 30 s 35 cycles 72 °C for 1 min 72 °C for 8 min 4°C forever
MatKF : 5' TAATTTACGATCAATTCATTC 3' MatKR : 5' ACAAGAAAGTCGAAGTAT 3'	94 °C for 2 min 94 °C for 30 s 52 °C for 30 s 35 cycles 72 °C for 1 min 72 °C for 8 min 4°C forever

Table 2: DNA quantification using Qubit Fluorometer

S. No.	Plant	DNA conc. µg/ml	Absorbance at A260/280
1	<i>Thottea siliquosa</i>	78.6	1.79
2	<i>Memecylon edule</i>	91.8	1.86
3	<i>Pajanelia longifolia</i>	80.5	1.81
4	<i>Bauhinia accuminata</i>	100	1.88
5	<i>Justicia betonica</i>	82.9	1.80

Figure 1: Genomic DNA of *Thottea siliquosa*, *Memecylon edule*, *Pajanelia longifolia*, *Bauhinia accuminata* and *Justicia betonica*

Figure 2.a: Gradient PCR Rbel primer

Figure 2.b: Gradient PCR RbcL primer

Figure 2.c: Gradient PCR RbcL primer

Figure 3: Amplification of *Thottea siliquosa*, *Memecylon edule*, *Pajanelia longifolia*, *Bauhinia accuminata* and *Justicia betonica* using RbcL primer

Figure 4: Amplification of *Thottea siliquosa* using MatK primer

Figure 5: Nucleotide BLAST analysis sequence of *Thottea siliquosa*

Figure 6: Nucleotide BLAST analysis sequence of *Memecylon edule*

Figure 7: Nucleotide BLAST analysis of *Bauhinia acuminata*

Figure 8: Nucleotide BLAST analysis of *Justicia betonica*

Figure 9: Nucleotide BLAST analysis of *Pajanelia longifolia*

CONCLUSION

DNA-based analysis helps in the identification of phytochemically indistinguishable genuine drug from a substitute drug. The sequence analysis of *Thottea siliquosa*, *Memecylon edule*, *Bauhinia accuminata* and *Justicia betonica* by BLASTN (NCBI) revealed 99 to 100 % nucleotide sequence homology with different database sequences of *Thottea siliquosa*, *Memecylon edule*, *Bauhinia accuminata* and *Justicia betonica*, thereby enabling species level confirmation. As sequence for longifolia species of genus *Pajanelia* or *Bignonia* was not available in the database and BLAST of analysis of *Pajanelia longifolia* sequence revealed 80 to 85 % sequence similarity with plant species belonging to genus *Bignonia*, only genus level confirmation was possible with *Pajanelia longifolia*. The sequences were submitted to Genbank database.

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