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ORIGINAL ARTICLE

DNA-based Simultaneous Identification of Three *Terminalia* Species Targeting Adulteration

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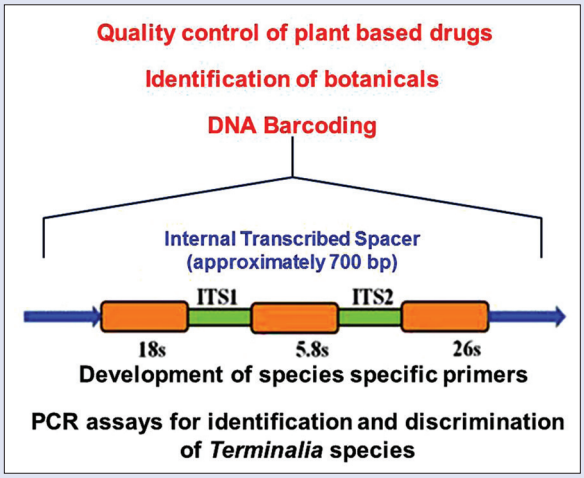
ABSTRACT

Background: Various parts of three *Terminalia* species, namely, *Terminalia arjuna* (stem bark), *Terminalia bellirica* (fruit), and *Terminalia chebula* (fruit) are widely known for their therapeutic principles and other commercial values. However, stem bark of *T. bellirica* and *T. chebula* along with *Terminalia tomentosa* are reported as adulterants of *T. arjuna*. Correct botanical identification is very critical for safe and effective herbal drugs. DNA-based identification approaches are advancing the conventional methods and sometime proved more beneficial. **Objective:** The purpose of the study was to develop polymerase chain reaction (PCR) method using internal transcribed spacer (ITS) region to ascertain the identity of *T. arjuna* herbal material as well as detection of mixing of other three *Terminalia* species. **Materials and Methods:** DNA from stem barks samples were isolated and subjected to ITS region amplification and sequencing. Sequences were compared for polymorphic nucleotides determination to develop species-specific primers. Final primers were selected on the basis of *in silico* analysis and experimentally validated. PCR assays for botanical identification of *Terminalia* species were developed. Sensitivity testing and assay validation were also performed. **Results:** The PCR assays developed for *Terminalia* species were resulted in definite amplicons of the corresponding species. No cross-reactivity of the primers was detected. Sensitivity was found enough to amplify as low as 2 ng of DNA. Mixing of DNA in various concentrations for validation also proved the sensitivity of assay to detect original botanicals in the mixture. The developed methods proved very specific and sensitive to authenticate Arjuna bark to develop evidence-based herbal medicines.

Key words: Botanical identification, internal transcribed spacer, polymerase chain reaction, stem bark, *Terminalia*

SUMMARY

- Internal transcribed spacer-based species-specific polymerase chain reaction (PCR) assays were developed to authenticate *Terminalia arjuna* stem bark and to identify substitution/adulteration of *Terminalia bellirica* and *Terminalia chebula* in the genuine starting material
- Definite amplicons were obtained specific to particular species and the assay was found of profound sensitivity to amplify as low as 2 ng of DNA
- Results of method validation proved that the assay can identify adulterant *Terminalia* species even when present in lower amounts
- The DNA barcodes and PCR methods can also be used to identify *Terminalia bellirica* and *T. chebula* related herbal medicinal material.



Abbreviations used: ITS: Internal transcribed spacer, BSA: Bovine serum albumin, DMSO: Dimethyl sulfoxide.

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INTRODUCTION

Bark of *Terminalia arjuna* (Roxb) Wight and Arn. and fruits of *Terminalia bellirica* (Gaertn.) Roxb. as well as *Terminalia chebula* Retz. , are well known for their therapeutic uses in Ayurveda.[1-3] All the three tree species belong to the family *Combretaceae*. Most prominently, curative effects of *T. arjuna* are clinically proven for cardiovascular and hepatic disorders.[1] Triterpenoids present in the stem bark of *T. arjuna* are cardioprotective whereas tannins and flavonoids are responsible for anticancer properties.[4] In addition to therapeutic values, all these tree species are also known for their significant role in timber, tasar silk, dyeing, leather, paper, resin, and soap industries.[5] However, stem barks of *T. bellirica* and *T. chebula* along with *Terminalia tomentosa* Wight and Arn. have also been reported as potent adulterants of *T. arjuna* bark, in order to attain high demands of arjuna-based drugs in the ayurvedic and herbal market.[6,7] Use of these three Terminalia species as the filler material in *T. arjuna* can reduce its curative effects and can pose serious health risk on consumers. Although, few therapeutic activities of the stem bark of these three *Terminalia* species have been reported, but, they are not extensively evaluated for any accountable therapeutic value similar to *T. arjuna*.[8] In addition, chemical composition of these stem bark different form Arjuna bark, can work synergistically or antagonistically with the bioactive principles of *T. arjuna*. Thus, adulteration and

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mixing of these three species can significantly affect commercialization of *T. arjuna*-based drugs. Genome-based identification methods either can facilitate the conventional macroscopy, microscopy, and chemical profiling techniques to develop a comprehensive approach for authentication and scientific validation of a species or can work as standalone technique.[9,10] In addition, DNA-based methods are more stable as seasonal, environmental, and age-related factors do not affect the reliability of results. They are also good to identify processed material in which taxonomic key characters are in reduced form.[11] In the present study, polymerase chain reaction (PCR) methods were developed to ascertain the identity of Arjuna bark raw material and to detect it along with cogeneric adulterants, in mixed herbal material. PCR methods were validated for specificity and sensitivity. PCR methods were developed using species-specific polymorphic sites present in the internal transcribed spacer region of plant nuclear genome. This region is highly polymorphic and high copies are present in the nuclear genome, hence has potential to be amplified in degraded biological material also.[12,13]

MATERIALS AND METHODS

Plant collection, DNA isolation, amplification, and sequencing of internal transcribed spacer locus

Stem bark samples of *Terminalia* species were collected from different locations of India and authenticated by conventional methods with the help of taxonomists. Voucher specimens with their specimen numbers were submitted in herbarium division of our institute. DNA isolated from bark samples was used for amplification of internal transcribed spacer (ITS) locus with the help of universal primers[14] using method previously reported by Sharma *et al.*[15] The amplified products were sequenced, edited, and assembled with the help of Sequence Analysis version 5.2 (Applied Biosystems, CA, USA) and Codon Code Aligner (Trial version, Codon Code, Dedham, MA, USA). Basic Local Alignment Search Tool (BLAST) was performed for verification of assembled contigs and sequence boundaries were determined comparing the sequences with the database.[16]

Selection of plant-specific primers

ITS sequences of all the four species were aligned with Clustal W tool using MEGA 5.1 to determine polymorphic nucleotides which were used further to develop species-specific primers.[17] Each site was checked manually to develop primers. *In silico* tools, OligoCalc and Primer3 softwares were used to test each polymorphic site for its feasibility to design suitable primers on the basis of optimal length, optimum GC content, melting temperature compatibility, hairpin formation, secondary structure, and species specificity through primer blast.[18,19] Bioinformatically tested primers were validated experimentally. ITS sequence comparison revealed absence of any polymorphic site between *T. arjuna* and *T. tomentosa* for suitable primer development of *T. tomentosa* while many polymorphic sites were present between and among other three *Terminalia* species. One forward and reverse primer were developed for authentication of *T. arjuna*, and primers for *T. bellirica* and *T. chebula* were also developed aiming the detection of the adulterants [Table 1].

Polymerase chain reaction assay for authentication of *Terminalia arjuna* stem bark

Primer pair TAF and TAR [Table 1] was used to develop Arjuna-specific identification method. Specificity of the primers and the reactions were evaluated by amplifying DNA of other two species with Arjuna-specific primers. Sensitivity of the method was tested using a range of dilutions of DNA from each species (2, 5, 10, 25, 50, 75, and 100 ng). DNA from stem bark samples of each species collected from various locations were isolated and amplified for validation. The reaction was conducted as follows - 1X Taq buffer, 2.50 mM $MgCl_2$, 200 μM dNTP, primers TAF and TAR 20 pm each, Taq Polymerase 1U, bovine serum albumin (BSA) 0.5 μg/μl following the program 94°C (10 min), 30 cycles of 94°C for (1 min), 60°C (45 s), 72°C (1 min), and final extension 72°C (5 min).

Development of multiplex polymerase chain reaction assay targeting adulteration detection in *Terminalia arjuna*, sensitivity testing, and validation

The developed species-specific primers were further used to develop a multiplex PCR assay to ensure the presence and absence of *T. arjuna* and its adulterant species in a mixture. To reduce the complexity, Arjuna-specific reverse primer (TAR) was dropped and ITS 4 was included as the common reverse primer. The experiment of multiplexing was designed with eight sets of reactions having different combination of primers and DNA template [Table 2]. Sensitivity of the developed method was assessed using a battery of DNA concentrations. Subsequently, DNA of three species was mixed in various ratios (total DNA concentration was approximately 50–60 ng) and subjected to amplification with multiplex PCR assay. The reaction was conducted using PCR components at the concentration of 0.75X Taq buffer, 2.50 mM $MgCl_2$, 400 μM dNTP, 20 pm TAF [Table 1], 1.5 pm TBF [Table 1], 10 pm TCF [Table 1], 15 pm ITS 4, 2U Taq Polymerase, 0.5 μg/μl BSA, 5% dimethyl sulfoxide (DMSO) following the program 94°C (10 min), 35 cycles of 94°C (1 min), 50°C (45 s), 65°C (1 min), and final extension 65°C (5 min).

RESULTS AND DISCUSSION

In the fast-growing market of plant-based medicine, quality and efficacy of herbal drugs are still a big bottle neck.[20] Quality parameters are negatively affected due to incorrect identification of the plant species, adulteration as well as nonscientific method of collection in inapt season. Application of DNA-based species identification method is widely growing which can be used individually or can facilitate the conventional approaches for authenticity detection. Moreover, DNA sequence-based approaches enhance the universality, accuracy, and precision in comparison to random DNA fingerprinting techniques. Instantaneously, sequence-inspired methods as simple sequence repeat, sequence-characterized regions, and DNA barcoding are resulted in better effectiveness to economic ratio.[21] Moreover, with advent of NGS technologies, genome-based identification of plant material has become more rapid and vast.[22]

Table 1: Sequence of the primers developed for molecular identification of *Terminalia* species

Species name	Primer name	Primer sequence (5′-3′)	Length (bp)	Tm	CG%	Approximate product size
Terminalia arjuna	TAF	GCCAAGGTACTCCAACGGAGG	20	62	60	269 bp with TAR and 517 bp with ITS 4
	TAR	GGAGGGTTTGGAGGCACGAT	20	55	60	
Terminalia bellirica	TBF150	GTCGATCTAAGCCCCAGCAG	20	58	60	150 bp
Terminalia chebula	TCF244	GATGGGAGGATGGTCCGGGA	20	55	65	244 bp

ITS: Internal transcribed spacer

Table 2: Summary of multiplex method development, validation, and results

Method development				
Reaction number	DNA added	Amplicons of corresponding species (bp)		
		517	244	150
1	TA	+	–	–
2	TB	–	–	+
3	TC	–	+	–
4	TA + TB	+	-	+
5	TB + TC	–	+	+
6	TA + TC	+	+	–
7	TA + TB + TC	+	+	+
8	NTC		–	
Method validation				
Ratio of DNA of TA:TB:TC		Amplicons of corresponding species		
		TA (517 bp)	TB (150 bp)	TC (244 bp)
1:1:1		+	+	+
2:1:1		+	+	+
1:2:1		+	+	+
1:1:2		+	+	+
4:1:1		+	+	+
1:4:1		+	+	+
1:1:4		+	+	+
8:1:1		+	–	–
1:8:1		+	+	+
1:1:8		+	–	+

TA: *Terminalia arjuna*; TB: *Terminalia bellirica*; TC: *Terminalia chebula*; NTC: No template control; "+" and "–": Positive PCR results or no any amplification, respectively; PCR: Polymerase chain reaction

Table 3: Locations of plant sample collection

Species name	Locations
Terminalia arjuna	Ahmedabad, Anand, Dang, DMAPR*, Gandhinagar, Junagarh, Nasik, Pune, Rajpipla, Udaipur
Terminalia tomentosa	Junagarh, Rajpipla, Udaipur
Terminalia bellirica	Ahmedabad, Anand, Gandhinagar, Pune, Rajpipla, Udaipur
Terminalia chebula	Anand, Gandhinagar, Pune, Rajpipla, Udaipur

*Directorate of medicinal and aromatic plant research, Anand

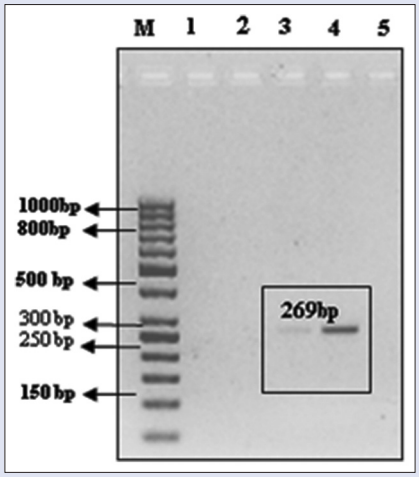

Figure 1: Two percent agarose gel is showing results of species-specific singleplex polymerase chain reaction assay developed for identification of *Terminalia arjuna*. Lane 1: *Terminalia chebula*; Lane 2: *Terminalia bellirica*; Lane 3: *Terminalia tomentosa*; Lane 4: *Terminalia arjuna*; and Lane 5: No template control; M: GeneRuler 50bp DNA Ladder (Thermo Scientific, Massachuetts, United States)

Plant collection, DNA isolation, amplification, and sequencing of internal transcribed spacer locus

To develop ITS-based identification method of *T. arjuna* and its adulterant, stem bark samples of all the four species were collected from various parts of the country [Table 3]. ITS region was amplified in all the species after DNA isolation from the collected bark samples.[15] The amplified ITS regions resulted in approximately 700 bp amplicon which was subsequently sequenced. Assembly of raw reads of sequence produced contigs of 667 bp (GeneBank: KF925432.1), 681 bp (GeneBank: KT187391), 720 bp (GeneBank: KC602394.1), and 701 bp (GenBank: KC750922.1) for *T. arjuna* (Pune, vouchered BVPPERD/PP/0812/11), *T. tomentosa* (Udaipur, vouchered BVPPERD/PP/0113/02), *T. bellirica* (Anand, vouchered BVPPERD/PP/0511/13), and *T. chebula* (Anand, vouchered BVPPERD/PP/0511/14), respectively. BLAST analyses of the contigs proved authenticity of the assembled sequences.

Selection of plant-specific primers, development of polymerase chain reaction assay for *Terminalia arjuna* identification

Polymorphic sites were identified in aligned sequence [with Clustal W tool] which was further used to develop species-specific primers for each species [Table 1]. Sequence comparison of *Terminalia* species revealed high homology between ITS sequences of *T. arjuna* and *T. tomentosa*, hence specific primer differentiating these two species could not be developed using ITS sequence. Investigation of sequence information for other loci such as rbcL, psbA-trnH, and matK revealed that only matK region has polymorphic nucleotides between these two *Terminalia* species. The focus of our study is the Arjuna stem bark and being part of chloroplast region, there are less chances of amplification of plastid regions in stem bark samples, therefore PCR method was developed for *T. arjuna, T. bellirica*, and *T. chebula*. At, first stage, a singleplex method was developed to ascertain the identity of *T. arjuna*. The results showed the specificity of primers of *T. arjuna,* as other than *T. arjuna* and *T. tomentosa*, none of the *Terminalia* species

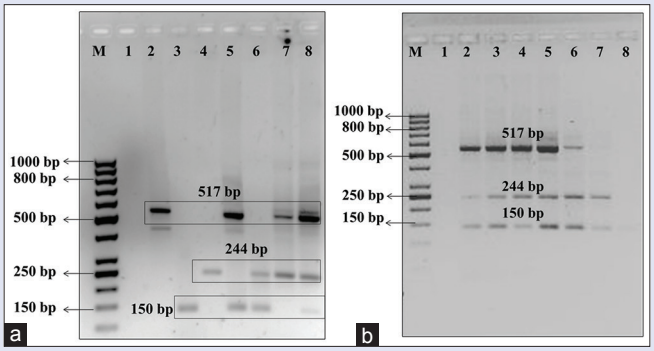

Figure 2: (a) Two percent agarose gel is showing results of multiplex polymerase chain reaction. Lane 2, Lane 3, and Lane 4 specific to *Terminalia arjuna*, *Terminalia bellirica*, and *Terminalia chebula*, respectively, in control reactions having all the three primers but DNA of only one species. Lanes 5, 6, and 7 are showing polymerase chain reaction results of control reactions having all three primers with DNA of two species. In Lane 8, all the three amplicons are found detectable upon mixing of DNA of all the three species. (b) Sensitivity testing of multiplex polymerase chain reaction assay. Lanes 2 to 8 are showing amplification from of each species having DNA concentrations 2, 5, 10, 25, 50, 75, and 100 ng, respectively. Lane 1: No template control; M: GeneRuler 50bp DNA Ladder (Thermo Scientific, Massachuetts, United States)

showed amplification. Specificity of the PCR assay was obtained by decrement in concentration of PCR components, especially primers and simultaneously annealing temperatures were increased with reduction in the annealing time. These approaches helped inhibit cross-amplification of the species and gave specific amplicons of 269 bp for *T. arjuna*. The reactions were found sensitive enough to amplify as low as 2 ng of DNA template. Universality of the primers and consistency of the PCR assays were validated through amplification of DNA from samples collected from various locations [Figure 1].

Development of multiplex polymerase chain reaction assay, sensitivity testing, and validation

Further, a multiplex reaction was developed utilizing the species-specific forward primers and a common reverse primer. Primers developed for *T. bellirica* and *T. chebula* were also validated experimentally in singleplex PCR. Arbitrary refractory mutation system technique was used to increase specificity of all these primers and subsequent assays.[23] Development of multiplex PCR for a sample having mixture of DNA from three different species using three different primers was found very challenging. Further, it was checked for for cross-reactivity and specificity also. Eight individual reactions were set up for the assay. Each reaction tube contained three forward primers (one each from individual *Terminalia* species) and one reverse primer along with other PCR components. These eight reaction tubes were further added with DNA templates of *Terminalia* species as per the experimental design mentioned in Table 2. Results of multiplex PCR assay [Table 2 and Figure 2] exhibited high specificity as all the eight PCR reactions amplified amplicons of very specific size corresponding with the *Terminalia* species was added. Addition of single species DNA resulted in a single band (517 bp for *T. arjuna*, 150 bp for *T. bellirica* and 244 bp for *T. chebula* in control reaction) while addition of two species combination generated two bands specific to the species added. In the reaction with three DNA templates, all the three bands were observed on the agarose gel. The result of multiplex assay showed the specificity of designed primer toward DNA of specific *Terminalia* species [Figure 2a and Table 2]. Development of this multiplex PCR assay claimed lots of optimization efforts.[24] Reduction in salt concentration in PCR buffer and higher concentration of TAF [Table 1] primer resulted in the appearance of *T. arjuna* band. Primer concentration for *T. bellirica* was needed to be reduced for appearance of band specific to *T. arjuna* and *T. chebula*. Five percent DMSO was added which increased the specificity with more clear bands on the agarose gel [Figure 2]. Some modifications in annealing and extension temperatures (from 72 to 65°C) led to more consistent PCR amplification.

Sensitivity of the multiplex assay was assessed using different concentrations of DNA template (2–100 ng) and was found to be sensitive to as low as 2 ng of DNA template of all the three *Terminalia* species when mixed in equal amount [Figure 2b].

Validation of multiplex PCR assay was done by mixing of DNA of *Terminalia* species in different ratios [Table 2]. Results of validation revealed that sensitivity of the assay to detect any *Terminalia* species was not affected much with changes in DNA amount ratios in DNA mixture containing all the *Terminalia* species.

CONCLUSION

Thus, PCR assays developed in this study are useful for identification of *T. arjuna*. Concurrently, detection of *T. bellirica* and *T. chebula* is possible if they are used as fillers in genuine raw material of *T. arjuna* bark. PCR methods developed in this investigation have the potential to identify and discriminate three *Terminalia* species with great specificity and sensitivity which can be further utilized as quality control for herbal drug starting material.

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Conflicts of interest

There are no conflicts of interest.

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