Original Research Article

Molecular authentication of Polyherbal formulation - Triphala churna

ABSTRACT

Aim: The present study aims to design a molecular authentication protocol for the renowned polyherbal formulation Triphalawith the help of recent advances in the DNA barcoding technology.

Purpose of work: In recent decades, ayurvedicpolyherbal medications have gained immense popularity worldwide. The quality of some of these herbal products has raised serious concerns regarding their authenticity and safety. The therapeutically potent original herbal species component of the polyherbal formulations are often substituted with cheaper or easily available adulterants or sometimes omitted entirely. In this case, molecular species authentication methods such as DNA barcoding can provide a far safer and efficient way to ensure species authenticity in polyherbal formulations.

Methodology: In the present study, ITS DNA barcoding based species authentication was performed for the polyherbal formulation – Triphala. For all the three component species of Triphala i.e. *Terminalia bellirica, Terminalia chebula*and *Phyllanthusemblica*, species authenticating primers were designed from their ITS barcoding regions and experimentally validated. The presented method was applied to authenticate all the three herbal species before they are blended in together to generate "Triphala" mixture as well as for quality assurance purpose for the already blended and processed formulation Triphala. This work also addresses the challenges in isolating DNA of satisfactory quality and quantity from highly acidic, polyphenolic, and commercially processed Triphalachurna samples after making some critical modifications in the traditional CTAB method; thus enabling their molecular authentication. Utilizing the designed species authenticating primers, a quality assurance inspection was conducted on seven Triphalachurna samples from reliable pharmacies that are available in the market.

Results: The present study successfully authenticates all the three ingredient species i.e. *Terminalia bellirica, Terminalia chebula*and*Phyllanthusemblica* in commercially available Triphalachurna samples with the help of ITS DNA barcoding technology.

Keywords: Barcoding; ITS, Molecular authentication; Polyherbal; Triphala

1. INTRODUCTION

According to World Health Organization (WHO), 60% of the world's population relies on herbal medicine and about 80% of the population in developing countries depends almost totally on it for their primary health care needs. [1]. As the global market for herbal medicinal products continues to grow, quality evaluation and standardization of herbal formulations are the fundamental requirements of pharmaceutical industries and other organizations dealing with ayurvedic and herbal products. Many substitutes and adulterants of original medicinal plants are now being traded because of their lower costs, easy availability or due to the misidentification of species with similar morphological features. Adulteration in market samples is one of the greatest drawbacks of promoting herbal products. adulterants or replacements are included in 4.2% of herbal medications sold [2].

Usually, the authentication of herbal ingredients relies on morphological, anatomical characteristics, and phytochemical profiling. However, the anatomical characteristics of similar species can be misleading in the identification of correct species and the chemical composition may be affected by growth, storage conditions, and harvesting process. In the past few decades, advances in molecular biology and Biotechnology have provided a more reliable approach for the authentication of herbal formulation that is identified on the DNA level. One such DNA based authentication technique is DNA barcoding. By combining the advantages of two molecular biology techniques—PCR amplification and sequencing—the DNA barcoding methodology provides a precise means of identifying species that have superficial physical similarities and are therefore difficult to distinguish using traditional taxonomy methods. The advent of DNA barcoding has made it possible to accurately identify materials with comparable morphologies and chemical structures and has helped herb identification enter the era of molecular identification, surpassing the limitations of old identification techniques [3]. A number of specific candidate regions, including matK, rbcL, psbA-trnH, and ITS, have been proposed for precise medicinal plant identification. Out of these, ITS region is chosen for species authentication in this study. Nuclear ribosomal DNA's internal transcribed spacer 2 (ITS2) region has a number of useful properties, including conserved regions that can be used to create universal primers, ease of amplification, and enough variability to allow for the differentiation of even closely related species [4]. Furthermore, ITS2 is potentially useful as a standard DNA barcode to identify medicinal plants [5].

The present study focuses on the species authentication of polyherbal formulation – Triphala. Triphala is one of the most popular polyherbal formulations to be used worldwide. It has various health benefits. Triphala is commonly used in Ayurvedic medicine to address gastric acidity, poor food absorption, constipation, and digestive issues. Furthermore, its application is suggested for the management of liver malfunction, obesity, anemia, weariness, inflammatory conditions, infections, and ocular issues. Triphala has cardio tonic properties; it increases blood circulation, lowers serum cholesterol and myocardial necrosis, and strengthens capillaries[6].Triphala is made up of three species i.e. *Terminalia bellirica*(Gaertn.) Roxb, *Terminalia chebula*(Retz.) Gaertn, and *Phyllanthusemblica L*..The taxonomic status of *Terminalia* species has been very controversial as they exhibit various overlapping morphotypes scattered in diverse regions and vary considerably in morphology and karyotype [7]. It is also reported that sometimes during the harvesting process of *Phyllanthusemblica*, traders mix chaff matter and other similar-looking fruits to increase the volume [8]. The market sample of Bhoi-amla botanically equated with *Phyllanthusamarus*is mixed with *Phyllanthusemblica* species[9]. Considering the immense popularity and market reach of Triphala, it is very much essential to ensure the safety, efficacy and efficiency of Triphalachurna products available in the market.

To authenticate the identity of *Terminalia bellirica, Terminalia chebula*and *Phyllanthusemblica,* species authenticating primers are designed from the ITS2 internal transcribed spacer barcoding region. These primers were experimentally validated for all three ingredient species of Triphalachurna. The designed primers were further successfully applied for the screening of local market-bought Triphalachurna samples for their quality control and species authentication purposes. This method successfully authenticates all the three component species of triphala individually before they are combined together to make Triphala formulation. Therefore, if adulteration occurs accidentally, it can be avoided before combining all the three herbs. The technique is further refined to detect any instances of species substitution, adulteration, or omission after the combination medicine Triphala is commercialized in the market in its processed form.

2. MATERIAL AND METHODS

2.1. Plant collection and DNA isolation:

Plant samples were collected from the different geographical regions of India and were identified by a taxonomist as *Terminalia bellirica, Terminalia chebula*and *Phyllanthusemblica*from anatomical characteristics. Location details of the collected samples are given in Table 1. Voucher specimens with their specimen numbers were submitted to Government Science College, Vankal, Gujarat, India. DNA was isolated by the CTAB method with modifications. The quantity and quality of the isolated DNA were assessed by 0.8% gel electrophoresis [10].

(Table 1: Location details of plant species collection sites)

Sr.no	Species name	Collection ID	Geographical location (lat and log)	Collection site
1.	*Terminalia bellirica*	TBD1	20°43'10.1"N 73°44'22.3"E	Daang forest
2	*Terminalia bellirica*	TBD2	20°41'14.7"N 73°47'39.9"E	
3	*Terminalia bellirica*	TBD3	20°36'39.3"N 73°46'49.9"E	
4	*Terminalia bellirica*	TBM1	17°55'38.7"N 73°38'30.0"E	Mahabaleshwar Forest
5	*Terminalia bellirica*	TBM2	17°55'29.0"N 73°37'37.0"E	
6	*Terminalia bellirica*	TBM3	17°55'26.6"N 73°40'32.5"E	
7	*Terminalia bellirica*	TBG1	23°13'16.0"N 72°40'42.0"E	Jawaharlal Nehru Herbal Botanical Garden, Gandhinagar
8	*Terminalia bellirica*	TBG2	23°13'18.6"N 72°40'37.4"E	
9	*Terminalia bellirica*	TBG3	23°13'14.0"N 72°40'48.0"E	
10	*Terminalia bellirica*	TBS1	21°04'06.9"N 72°53'38.1"E	Surat Nursery
11	*Terminalia chebula*	TCD1	20°51'42.2"N 73°33'23.5"E	Daang forest
12	*Terminalia chebula*	TCD2	20°49'16.7"N 73°35'30.0"E	
13	*Terminalia chebula*	TCD3	20°47'49.6"N 73°34'33.1"E	
14	*Terminalia chebula*	TCM1	17°56'03.8"N 73°38'51.3"E	Mahabaleshwar Forest
15	*Terminalia chebula*	TCM2	17°56'06.7"N 73°39'16.2"E	
16	*Terminalia chebula*	TCM3	17°55'53.0"N 73°39'07.9"E	
17	*Terminalia chebula*	TCG1	23°13'16.2"N 72°40'35.3"E	Jawaharlal Nehru Herbal Botanical
18	*Terminalia chebula*	TCG2	23°13'10.3"N 72°40'35.5"E	

19	*Terminalia chebula*	TCG3	23°13'20.7"N 72°40'40.1"E	Garden, Gandhinagar
20	*Terminalia chebula*	TCS1	21°04'07.5"N 72°53'38.7"E	Surat Nursery
21.	*Phyllanthusemblica*	PED1	20°56'40.8"N 73°44'18.5"E	Daang forest
22	*Phyllanthusemblica*	PED2	20°55'46.8"N 73°44'52.5"E	
23	*Phyllanthusemblica*	PED3	20°55'43.7"N 73°48'53.9"E	
24	*Phyllanthusemblica*	PEM1	17°56'04.5"N 73°38'48.1"E	Mahabaleshwar Forest
25	*Phyllanthusemblica*	PEM2	17°56'01.5"N 73°38'39.9"E	
26	*Phyllanthusemblica*	PEM3	17°55'51.3"N 73°38'20.2"E	
27	*Phyllanthusemblica*	PEG1	23°13'18.4"N 72°40'47.4"E	Jawaharlal Nehru Herbal Botanical Garden, Gandhinagar
28	*Phyllanthusemblica*	PEG2	23°13'14.7"N 72°40'47.7"E	
29	*Phyllanthusemblica*	PEG3	23°13'12.5"N 72°40'44.9"E	
30	*Phyllanthusemblica*	PES1	21°04'07.8"N 72°53'40.0"E	Surat Nursary

2.2. DNA barcoding using ITS primers:

PCR was carried out using EmerlandAmp®PCR Reaction Mix (TAKARA) and template DNA (50 ng/µL). The polymerase chain reaction was carried out in Thermalcycler (Applied Biosystems Veriti®) using ITS PCR Primers [11] ITS1(F) TCCGTAGGTGAACCTGCGG and ITS4(R) TCCTCCGCTTATTGATATGC with the following parameters: 95°C for 5 min; 35 cycles at 95°C for 60 seconds, 54°C for 30 seconds, and 72°C for60 seconds, and a final extension at 72°C for 10 min. PCR products were subjected to agarose gel (1.5% [w/v]) electrophoresis in 1 × TBE buffer, along with 100-bp DNA ladders (Banglore Genie, India) as size markers. DNA was stained with ethidium bromide and photographed under UV light. The amplified products were sequenced using 3500 XL Genetic Analyser (Applied Biosystems). The sequences were edited, and assembled using Chromas version 2.6.6. Basic Local Alignment Search Tool (BLAST) was performed for verification of assembled contigs [12].

2.3. **Designing of species specific primers:** For all three species, the obtained ITS barcoding sequence was aligned with the available sequences from BLAST using Clustal Omega software [12]. The common DNA regions among them were traced and were used for the design of species authenticating primers using the *in silico* tool Primer3 software. The primers were selected on the basis of optimal length, optimum GC content, melting temperature compatibility, hairpin formation, secondary structure. Bioinformatically tested primers were validated experimentally.

2.4. **Experimental validation of the designed primers with their designated species through Polymerase chain reaction:** Specificity and authentication ability of the designed primers were tested by performing PCR with each respective species i.e. *Terminalia bellirica, Terminalia chebula* and *Phyllanthusemblica.* The reaction was conducted as follows - PCR EmerlandAmp®PCR Reaction Mix (TAKARA) and template DNA (50 ng/µL)., Forward primers and reverse primes 0.2µM each for all the three species following the program 95°C (5 min), 30 cycles of denaturation at 95°C for (30 sec), annealing at 50°C, 58°C, 50°C for ITSTBC1F/ R, TCF244/ITS4, ITSPEC1F/ R respectively (45 s),

and extension at 72°C (1 min), followed by 1 cycle of final extension 72°C (5 min) [12].

2.5. Screening of commercially available Triphalachurna using self-designed species authenticating primers:

2.5.1. **Isolation of DNA from Triphalachurna:** Total seven Triphalachurna samples from reputed pharmacies were procured from the local ayurvedic pharmacy of Surat, Gujarat, India; and further processed for DNA extraction. The classic CTAB extraction protocol given by Doyle and Doyle, 1987 [13]. was modified to extract DNA from Triphala samples that are highly acidic and polyphenolic in nature. The ratio of CTAB extraction buffer volume to Triphalachurna sample was adjusted in such a way that the pH of cell lysate remains around 7.5-8. To deal with the high levels of polyphenolic content of *Terminalia bellirica, Terminalia chebula*and *Phyllanthusemblica,* the concentration of PVP (Polyvinylpyrrolidone) was increased upto 4% (W/V).Quantitative spectrophotometric assay of DNA was performed using a Nanodrop spectrophotometer. The integrity of genomic DNA was tested by resolving DNA extracts on a 0.8% agarose gel by electrophoresis (Bio-Rad, Hercules, CA, USA), followed by visualization with ethidium bromide staining. The isolated DNA samples were subjected to Polymerase Chain Reaction using the species authenticating primers for all three individual species; thereby confirming the presence of *Terminalia bellirica, Terminalia chebula,* and *Phyllanthusemblica* in the local Triphalachurna samples.

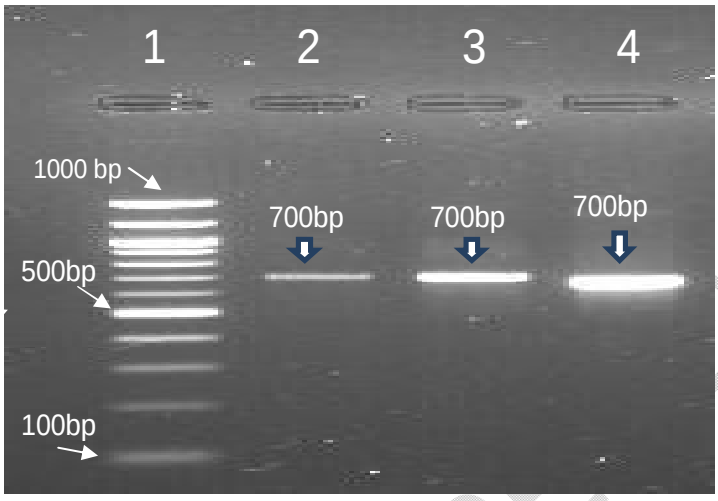
2.5.2. **Polymerase chain reaction assay for authentication of *Terminalia bellirica, Terminalia chebula,* and *Phyllanthusemblica*species in commercially available Triphalachurna samples:**The designed primer pairs were used to develop species identification method for their respective species. Specificity of the primers and the reactions were evaluated by amplifying DNA of commercially available Triphala samples of seven reputed ayurvedic pharmacies. The reaction was conducted as follows – PCR mastermixEmerlandAmp®PCR Reaction Mix (TAKARA), Forward primers and reverse primes 0.2µM each for all the three species, along with the 50 ng/µL DNA template. The PCR conditions for all seven commercially available Triphalachurna samples are as follows: 95°C (5 min), 30 cycles of denaturation at 95°C for (30 sec), annealing at 50°C, for ITSTBC1F/ R, ITSTCC1F/R, ITSPEC1F/ R (45 s), and extension at 72°C (1 min), followed by 1 cycle of final extension 72°C (5 min).To confirm the species identity, all the three amplicons obtained for their respective species, were sequenced [12].

2.5.3. **Sequencing of PCR amplicon obtained by designed primers for the confirmation of species identity:**The amplicon of one Tiphalachurna sample out of seven was chosen for sequencing with species identification purpose for *Terminalia bellirica, Terminalia chebula and Phyllanthusemblica ea*ch. Amplified PCR product was purified using BigDye® Terminator v3.1 Cycle Sequencing clean up method with some modification used further for sequencing. Data were retrieved from the sequencer and further analysed for similarity index using NCBI-BLASTN for the nomenclature of sequence and submitted to NCBI database using BankIt. Phylogenetic trees were constructed using full optimal alignment in the Clustal_X version 2.0 Software and neighbor-joining method with 1000 bootstrap replications available in the MEGA version 6.0 [14].

3. RESULTS AND DISCUSSION

3.1. Plant collection, DNA isolation, amplification, and sequencing of internal transcribed spacer locus: To develop

ITS‐based identification method for *Terminalia bellirica, Terminalia chebula* and *Phyllanthusemblica* leaf samples of all the three species were collected from Daang, Mahabaleshwar and Gandhinagar (Jawaharlal Nehru Herbal botanical Garden), India. ITS region was amplified in all the species after DNA isolation from the collected leaf samples. The amplified ITS regions resulted in approximately 700 bp amplicon which was subsequently sequenced. (Fig. 1)



(Fig. 1. Plant species DNA amplified with the universal ITS barcoding primer. Lane 1: 100 bp molecular marker, Lane 2: *Terminalia bellirica*DNA amplified with ITS primers, Lane 3: *Terminalia chebula*DNA amplified with ITS primers, Lane 4: *Phyllanthusemblica*DNA amplified with ITS primers)

Assembly of raw reads of sequence produced contigs of 684 bp (GeneBank:OP363978.1), 695 bp (GeneBank:OP363962.1) and 694 bp (GeneBank:OP389072.1), for *Phyllanthusemblica*, *Terminalia chebula* and *Terminalia bellirica* respectively. BLAST analysis of the contigs proved authenticity of the assembled sequences.

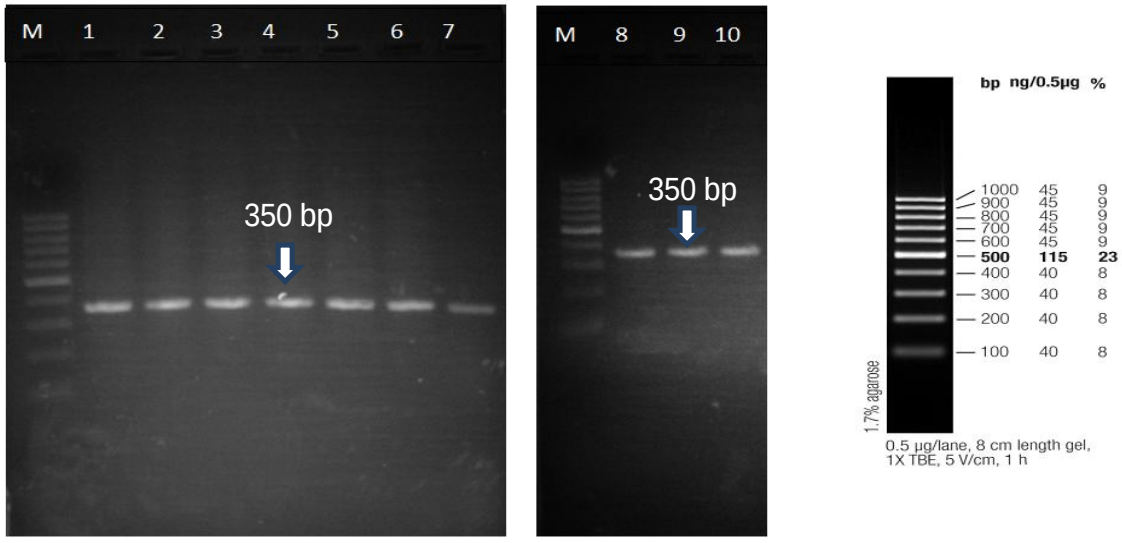
3.2. Selection of plant‐specific primers, development of polymerase chain reaction assay for *Terminalia bellirica, Terminalia chebula, Phyllanthusemblica*identification: Using Clustal Omega software, the acquired ITS barcoding sequence for each of the three species was matched with the sequences that were available from BLAST. The most prevalent DNA segments that were found to be present in all of them were identified and utilized to create the species authenticating primers. The list of species authenticating primers is given below in table 2.

Table 2: Species authenticating primers for *Terminalia bellirica, Terminalia chebula, Phyllanthusemblica*

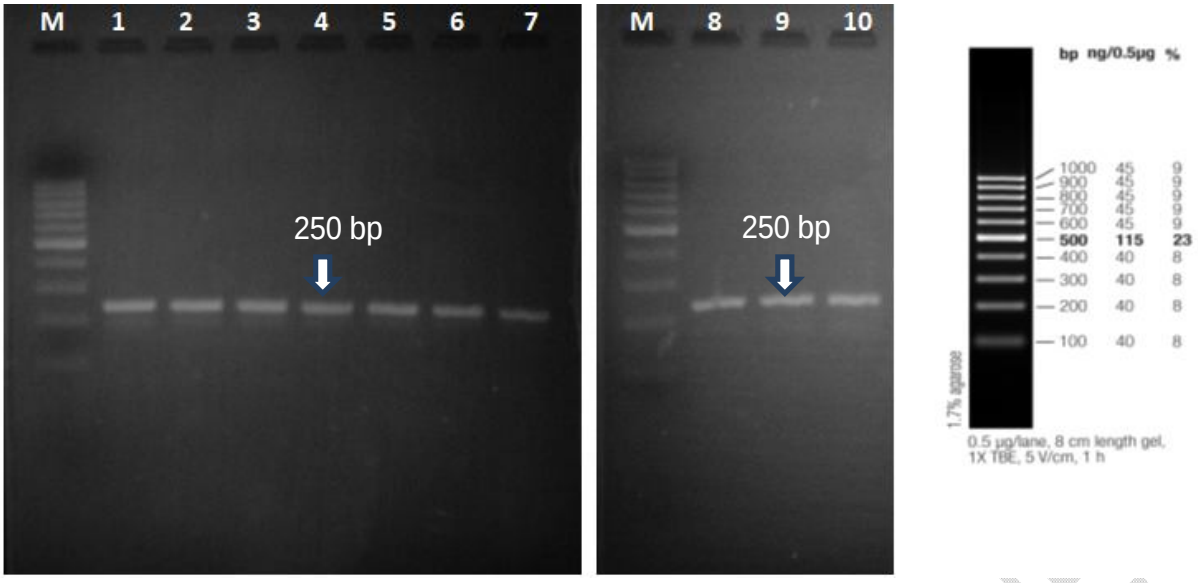
Species	Sample ID	Primer name	Sequence	Expected product size
Terminalia bellirica	TBD 1,2,3; TBM 1,2,3; TBG 1,2,3,	ITSTBC1F (Forward)	CATTGTCGGTACCTGCAA	354 bp
		ITSTBC1R	CGTTACTAAGGGAATCCTTGTA	

	TBS1	(Reverse)		
Terminalia chebula	TCD 1,2,3; TCM 1,2,3; TCG 1,2,3, TCS1	ITSTCC1F (Forward)	GAAGGATCATTGTCGATACCT	240 bp
		ITSTCC1R (Reverse)	CGTTCAAAGACTCGATGGT	
Phyllanthus emblica	PED 1,2,3; PEM 1,2,3; PEG 1,2,3, PES1	ITSPEC1R (Forward)	GAAGTCCACTGAACCTTATCAT	755 bp
		ITSPEC1R (Forward)	CGTTACTAAGGGAATCCTTGTA	

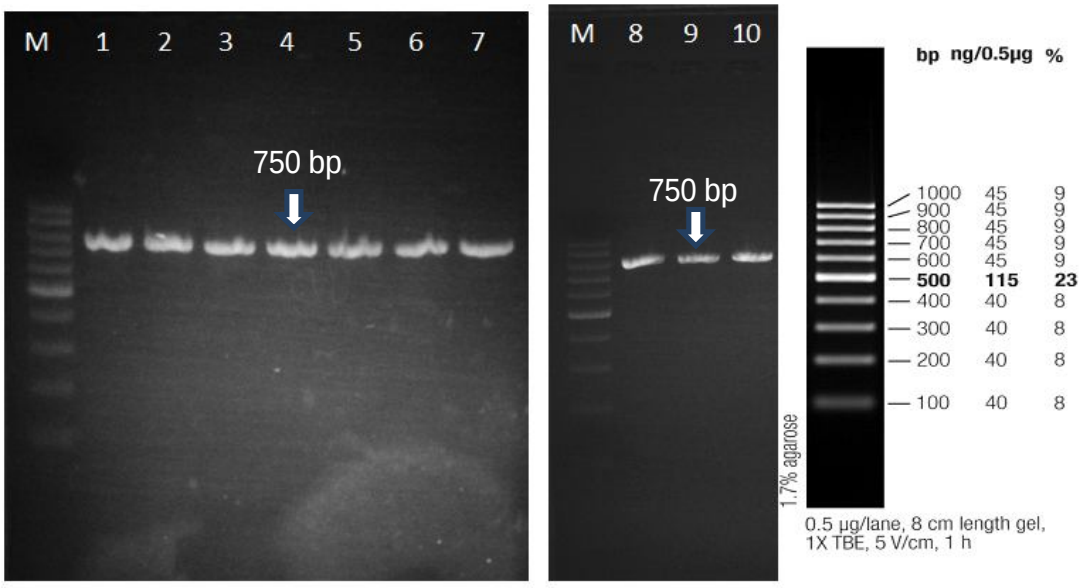
The developed primers mentioned in table 2 gave successful amplification with their respective plant species collected from different geographical regions of Gujarat and Maharashtra, Surat. (Figure 2, Figure 3, Figure 4).



(Fig. 2.DNA samples of *Terminalia bellirica* species,amplified with the primer ITSTBC1F/R. Lane M: 100 bp DNA ladder; Lane 1,2,3: *Terminalia bellirica* leaf samples collected from Daang, Lane 4,5,6: : *Terminalia bellirica* leaf samples collected from Mahabaleshwar; Lane 7, 8,9,10: *Terminalia bellirica* leaf samples collected from Gandhinagar, Surat)



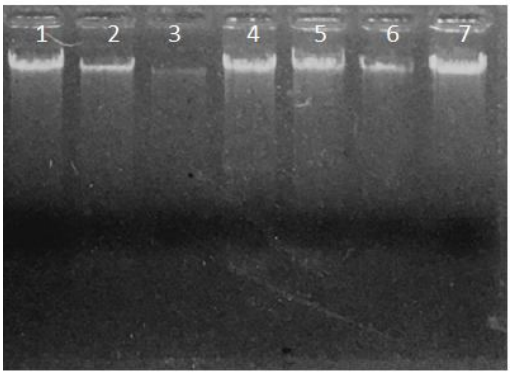
(Fig. 3. DNA samples of *Terminalia chebula* species,amplified with the primer ITSTCC1F/R. Lane M: 100 bp DNA ladder; Lane 1,2,3: *Terminalia chebula* leaf samples collected from Daang, Lane 4,5,6:*Terminalia chebula* leaf samples collected from Mahabaleshwar; Lane 7, 8,9,10: leaf samples collected from Gandhinagar, Surat)



(Fig. 4. DNA samples of *Phyllanthus emblica* speciesamplified with the primer ITSPEC1F/R. Lane M: 100 bp DNA ladder; Lane 1,2,3: *Phyllanthue emblica* leaf samples collected from Daang, Lane 4,5,6: *Phyllanthue emblica* leaf samples collected from Mahabaleshwar; Lane 7, 8,9,10: *Phyllanthue emblica* leaf samples collected from Gandhinagar, Surat)

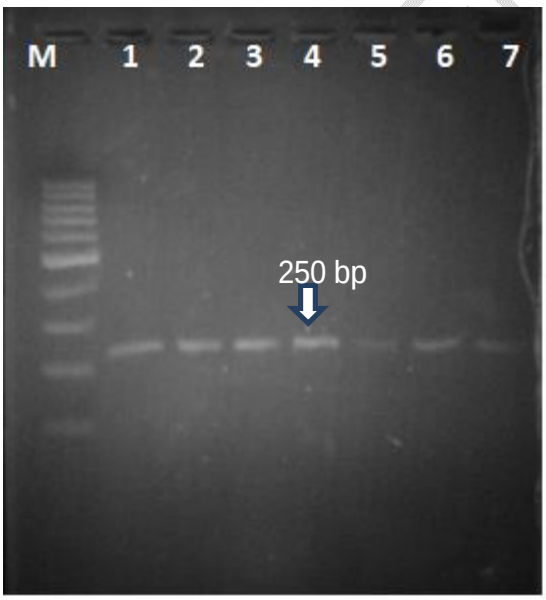
The primers designed to authenticate the species *Terminalia bellirica, Terminalia chebula* and *Phyllanthus emblica* produced the amplicons of size of approximately 350 bp, 250 bp and 750 bp respectively in all the three plant species samples collected from different geographical regions of India proving their competence for species authentication.

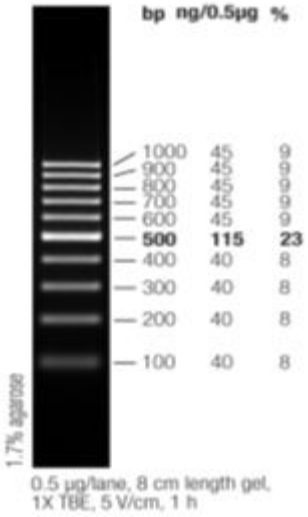
3.3. **Screening of commercially available Triphala churna samples using the designed species authenticating primers:** Total seven Triphala churna samples from reputed aayurvedic pharmacies were screened with the species authenticating primers designed from ITS barcoding regions. The first crucial step for this was successful DNA isolation from churna samples. The traditional CTAB method was modified to deal with the highly polyphenoloc and acidic nature of the component species. The DNA samples isolated were checked with the help of 0.8% agarose gel electrophoresis and were found to be of satisfactory quality (Figure 5). This procedure yielded DNA of satisfactory concentration from all the seven commercial triphala samples upon evaluated by using a Nanodrop spectrophotometer. All the samples gave A260/A280 ratio between 1.6-1.9.



(**Fig. 5. Agarose gel electrophpresis image of DNA isolated from commercially available Triphala churna samples.** Lane 1 to 7: Isolated DNA from Triphala churna samples TCS1 to TCS7)

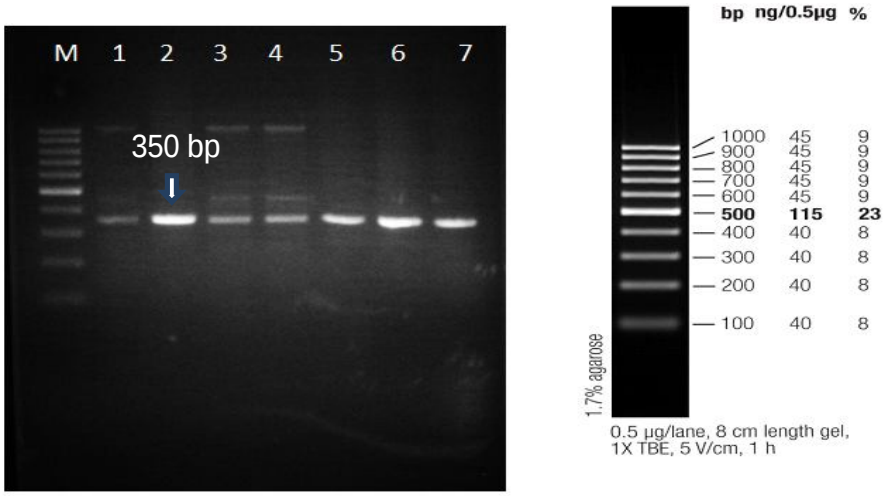
3.4. **Screening of commercially available Triphala churna samples using species authenticating primers:**All seven Triphala churna samples from reputed pharacies TCS1 to 7 were screened for species authentication using self designed primers. All seven samples gave successful amplification for the primers designed for *Terminalia chebula* i.e. ITSTCC1F/R and gave the expected amplicon of size of approximately 250 bp. Hereby confirmng the presence of *Terminalia chebula* in all the seven Triphala samples (Figure 6).





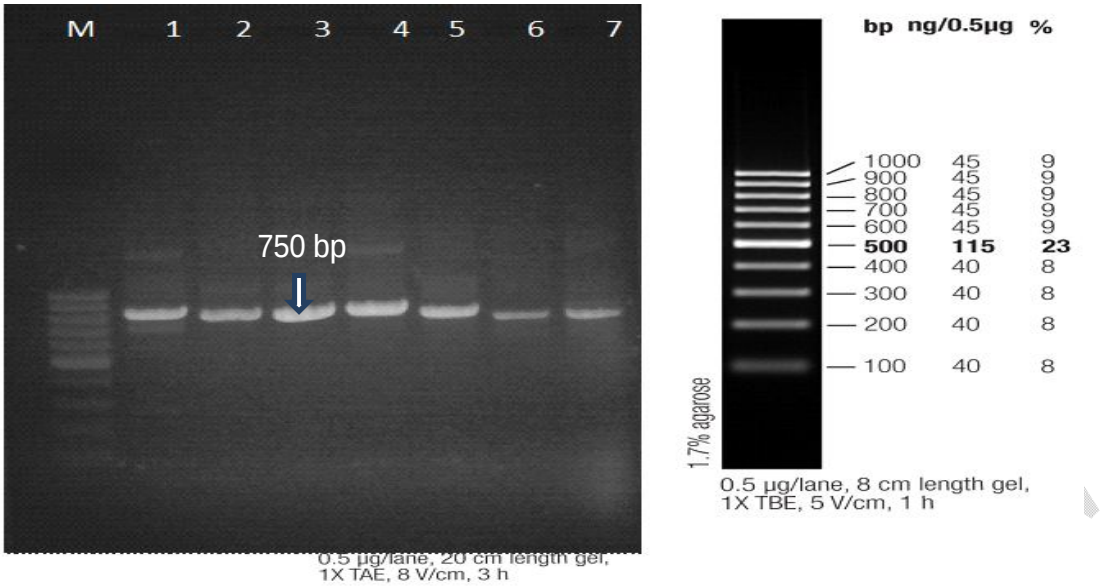
(Fig. 6. Gel electrophoresis image of commercially available Triphalachurna samples TCS 1 to 7 successfully amplified by *Terminalia chebula* species authenticating primer pair ITSTCC1F/R. Lane M: DNA ladder; Lane 1 to 7: Triphalachurna samples TCS 1 to 7 successfully amplified by species authenticating primer pair ITSTCC1F/R)

All seven samples gave successful amplification for the primers designed for *Terminalia bellirica* i.e. ITSTBC1F/R and gave the expected amplicon of size of approximately 350 bp. Along with the species authenticating band of expected size, there are some additional bands suggesting the presence of other contaminants or species admixtures in the Triphala churna samples.



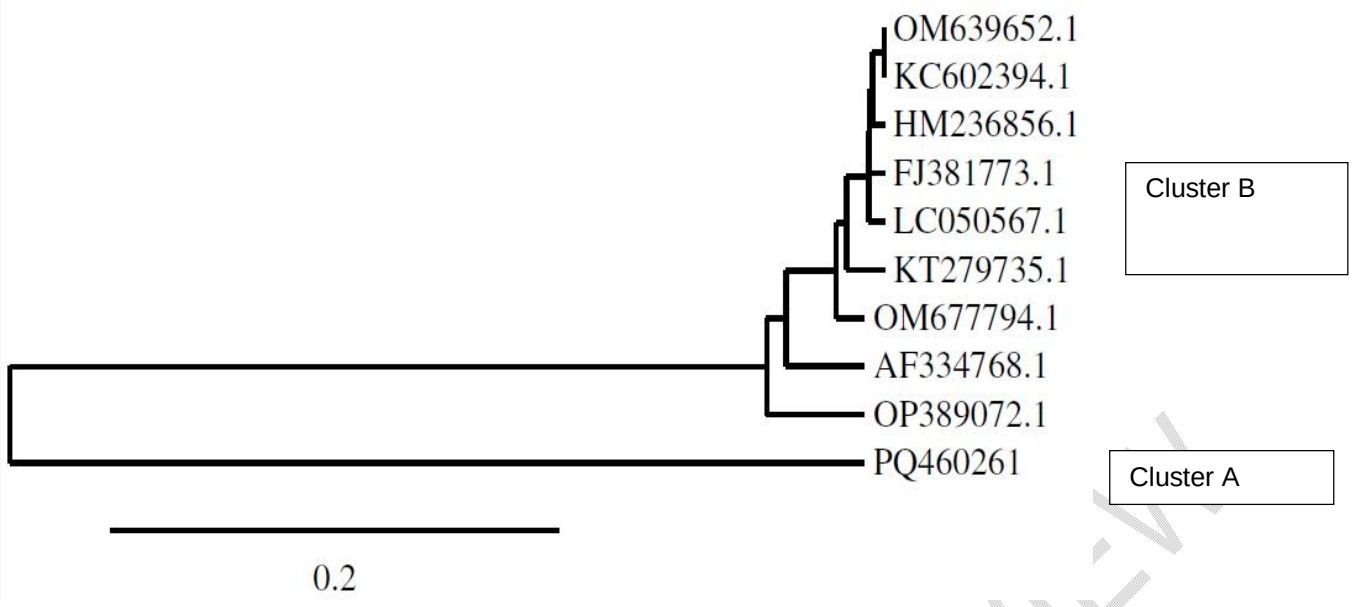
(Fig. 7. Gel electrophoresis image of commercially available Triphalachurna samples TCS 1 to 7 successfully amplified by *Terminalia bellirica* species authenticating primer pair ITSTBC1F/R. Lane M: DNA ladder; Lane 1 to 7: Triphalachurna samples TCS 1 to 7 successfully amplified by species authenticating primer pair ITSTBC1F/R)

All seven samples gave successful amplification for the primers designed for *Phyllanthus emblica* i.e ITSPEC1F/R and gave the expected amplicon of size of approximately 750 bp (Figure 8). Along with the species authenticating band of expected size, there are some additional bands suggesting the presence of other contaminants or species admixtures in the Triphala churna samples.



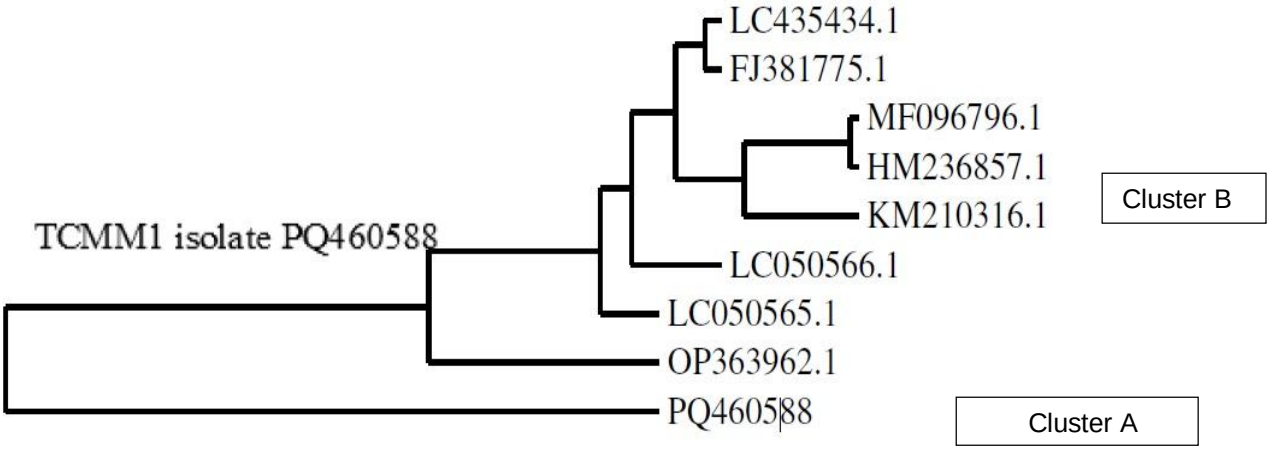
(Fig. 8. Gel electrophoresis image of commercially available Triphalachurna samples TCS 1 to 7 successfully amplified by *Phyllanthusemblica*species authenticating primer pair ITSPEC1F/R. Lane M: DNA ladder; Lane 1 to 7: Triphalachurna samples TCS 1 to 7 successfully amplified by species authenticating primer pair ITSPEC1F/R)

3.5. **Sequencing of PCR amplicon obtained by designed primers for the confirmation of species identity:** The amplicons obtained of approximately 350 bp, 250 bp and 750 bp by PCR reaction of Triphalachurna DNA with species authenticating primers designed for *T. bellirica, T. chebula* and *P. emblica* respectively were sequenced by Sanger sequencing. Upon sequencing, the exact size of amplicons were found to be 354 bp, 240 bp and 755 bp with the designed primers ITSTBC1F/R, ITSTCC1F/R and ITSPEC1F/R respectively. These sequences were subjected to nucleotide BLAST to assess the homology with the available sequences of the database. Upon BLAST analysis, sequence of amplicon sized 354 bp amplified with primer ITSTBC1F/R showed 100% query cover and 99.68% similarity with *Terminalia bellirica* isolate TBMM1 small subunit ribosomal RNA gene, partial sequence (OP389072.1). Considering the similarity in BLAST results upto 98.71%, the sequence was named as *Terminalia bellirica*TBMM1 isolate and deposited in the genebank with excession number PQ460261. This *Terminalia bellirica* TBMM1 isolate PQ460261 was compared to other NCBI sequences for multiple sequence analysis using CLUSTAL Omega software and neighbour joining method with 1000 bootstrap replications followed by a phylogenetic tree construction using neighbour joining method of MEGA 6 [14]. Phylogenetic analysis evidenced that *Terminalia bellirica* TBMM1 isolate PQ460261 belongs to different cluster A, related to other isolates, which belong to the same cluster B. These data revealed that *Terminalia bellirica* TBMM1 isolate PQ460261 evolutionary differ from other sequences in cluster. The phylogenetic tree is shown in figure 9.



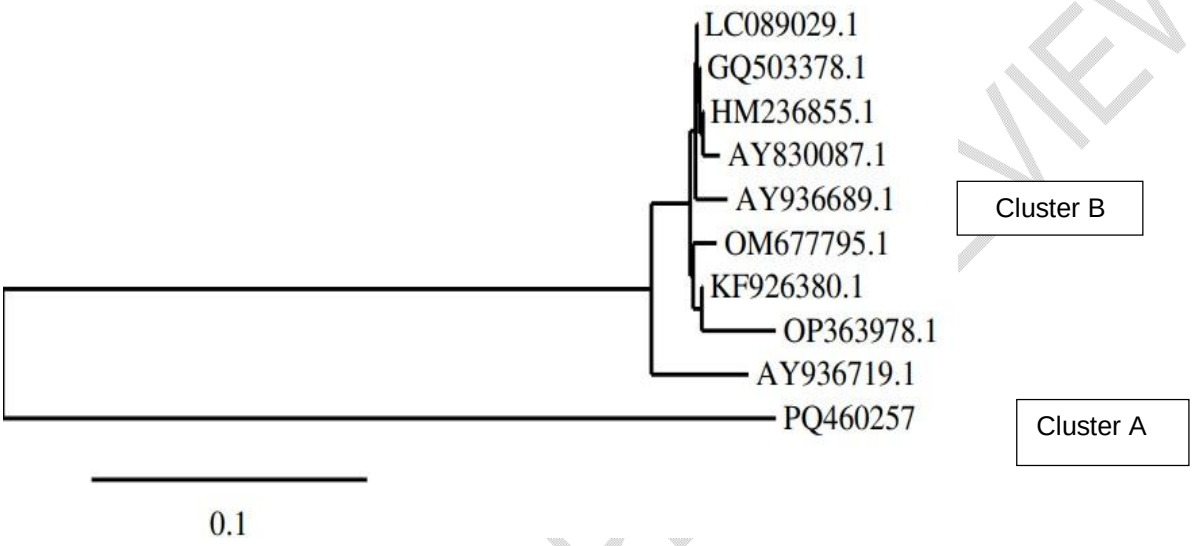
(Fig. 9. Phylogenetic tree showing the genetic relationship between *Terminalia bellirica* TBMM1 isolate PQ460261 with other isolates from NCBI genebank).

Upon BLAST analysis, sequence of amplicon sized 240 bp amplified with primer ITSTCC1F/R showed 100% query cover and 97.84% similarity with *Terminalia chebula* isolate TCMM1 small subunit ribosomal RNA gene, partial sequence (KM210316.1). Considering the similarity in BLAST results upto 92%, the sequence was named as *Terminalia chebula* TCMM1 isolate and deposited in the genebank with excession number PQ460588. This *Terminalia chebula* TCMM1 isolate PQ460588 was compared to other NCBI sequences FOR multiple sequence analysis using CLUSTAL Omega software and neighbour joining method with 1000 bootstrap replications followed by a phylogenetic tree construction using neighbour joining method of MEGA 6 [14]. Phylogenetic analysis evidenced that *Terminalia chebula* TCMM1 isolate PQ460588 belongs to different cluster A, related to other isolates, which belong to the same cluster B. These data revealed that *Terminalia chebula* TCMM1 isolate PQ460588 evolutionary differ from other sequences in cluster. The phylogenetic tree is shown in figure 10.



(Fig. 10. Phylogenetic tree showing the genetic relationship between *Terminalia chebula* TCMM1 isolate PQ460588 with other isolates from NCBI genebank).

Upon BLAST analysis, sequence of amplicon sized 755 bp amplified with primer ITSPEC1F/R showed 100% query cover and 99.71%% similarity with *Phyllanthusemblica* isolate PEMM1 small subunit ribosomal RNA gene, partial sequence (AY830087.1). Considering the similarity in BLAST results upto 98.65%, the sequence was named as *Phyllanthusemblica*PEMM1 isolate and deposited in the genebank with excession number *PQ460257*. This *Phyllanthusemblica*PEMM1 isolate PQ460257 was compared to other NCBI sequences for multiple sequence analysis using CLUSTAL Omega software and neighbour joining method with 1000 bootstrap replications followed by a phylogenetic tree construction using neighbour joining method of MEGA 6 [14]. Phylogenetic analysis evidenced that *Phyllanthusemblica*PEMM1 isolate belongs to different cluster A, related to other isolates, which belong to the same cluster B. These data revealed that *Phyllanthusemblica*PEMM1 isolate evolutionary differ from other sequences in cluster. The phylogenetic tree is shown in figure 11.



(Fig. 11. Phylogenetic tree showing the genetic relationship between *Terminalia chebula* TCMM1 isolate PQ460588 with other isolates from NCBI genebank).

From the above results, identity of *Terminalia bellirica, Terminalia chebula and Phyllanthusemblica* were confirmed in the Triphalachurna samples TCS1 to TCS7, hence ensuring the authenticity of these samples.

4. DISCUSSION:

The designed primers were successful for the authentication of their designated species in control plant samples as well as in the processed churnasamples.The first major challenge of the study was the successful DNA isolation from the Triphalachurna samples. Many previously established protocols of DNA isolation such as Doyle and Doyle (1997) [13], Murray and Thompson (1980) [23], Warude*et al;* (2003) [24] were unsuccessful in isolating pure and PCR amplifiable DNA from churna samples. All The three ingredients of Triphala i.e. *P. emblica*, *T. bellirica*, and *T. chebula* are highly acidic in nature. When the churna sample is treated with extraction buffer, due to the acidic nature of the component species, the pH drops to 2-3. In this pH, the extracted DNA will be degraded. By adjusting the volume of extraction buffer to adjust the pH to 7.5-8.0, the DNA was observed to be in intact form. Moreover the concentration of PVP has also been increased to remove the polyphenolic components present in the component species. The additional C:I

(Chloroform:isoamyl alcohol) steps were performed to remove all the protein impurities from the DNA sample. This protocol yields DNA of satisfactory quantity and quality.

Once, the successful DNA isolation was carried out, the DNA barcoding for the each species was carried out using the universal ITS primers. The obtained ITS DNA barcodes for all three species were utilized for the design of species authenticating primers. For each species, the DNA barcoding sequence was aligned with the most closely related sequences of the same species retrieved from NCBI BLAST. Using Clustal Omega bioinformatics tool [12], the most ubiquitous DNA regions among the aligned sequences of a species were traced. These DNA regions were subjected to Primer BLAST tool for the design of primers having the potential to authenticate the corresponding species. Among many potential primer candidates, three primer pairs ITSTBC1F/R, ITSTCC1F/R and ITSPEC1F/R were selected for *Terminalia bellirica, Terminalia chebula*and*Phyllanthusemblic*a. These designed primers were subjected to wet lab validation to assess their species authenticating potential. The primers were subjected to PCR amplification with ten plant samples per species collected from different geographical locations of India (Table 1). Primer ITSTBC1F/R gave successful amplification with the same product size of around 350 bp in all the ten plant samples of *Terminalia bellirica* species collected from different geographical locations of India. Likewise, Primer ITSTCC1F/R gave successful amplification with the same product size of around 250 bp in all the ten plant samples of *Terminalia chebula* species collected from different geographical locations of India. In case of primer ITSPEC1F/R gave successful amplification with the same product size of around 750 bp in all the ten collected plant samples of *Phyllanthusemblica* species.

After successful experimental validation, primers ITSTBC1F/R, ITSTCC1F/R and ITSPEC1F/R were assigned as species authenticating primers for *Terminalia bellirica, Terminalia chebula*and *Phyllanthusemblica* respectively. Before the blending of these three species into Triphalachurna formulation, the designed primers can be used to ensure the identity of the component species. The designed primers were further scrutinized to assess their species authenticating potential in the seven processed, powdered, commercially available Triphalachurna samples procured from Surat and surrounding areas(TCS1 to TCS7). Even in the processed Triphalachurna the primers ITSTBC1F/R, ITSTCC1F/R and ITSPEC1F/R gave the exact same sized amplicons as they did with control plant samples representing their corresponding species in the mixture. Thus the designed ITSTBC1F/R, ITSTCC1F/R and ITSPEC1F/R primers were found to be successful in authenticating their corresponding species i.e. *Terminalia bellirica, Terminalia chebula*and *Phyllanthusemblica* respectively in the processed Triphalachurna samples (Figure 6, Figure 7, Figure 8).

To further ensure the identity of *Terminalia bellirica, Terminalia chebula*and *Phyllanthusemblica,* the amplicons obtained for each species with Triphala DNA were subjected to Sangar sequencing. The sequences of amplicons 354 bp, 240 bp and 755 bp were subjected to NCBI BLAST to analyse their homology with the available sequences of the database. Moreover, Phylogenetic tree by NJ method were constructed to further confirm the species identity of all the amplicon sequences [14]. From the above analysis, the amplicon of 354 bp obtained by PCR reaction of Triphalachurna DNA with ITSTBC1F/R primer was identified as the product of *Terminalia bellirica* DNA present in the Triphalachurna samples. Likewise, the amplicon of 240 bp obtained by PCR reaction of Triphalachurna DNA with ITSTCC1F/R primer was identified as the product of *Terminalia chebula* DNA present in the Triphalachurna samples. The amplicon of 755 bp obtained by PCR reaction of Triphalachurna DNA with ITSTBC1F/R primer was identified as the product of *Phyllanthusemblica*DNA present in the Triphalachurna samples.

However, when Triphalachurna DNA was amplified using primers ITSTBC1F/R and ITSPEC1F/R, a number of extra unintended DNA bands were also observed. These bands were not present when *Terminalia bellirica* and *Phyllanthusemblica* control plant DNA was amplified using the same primers (Figure 2, 4). This indicates the possibility of

presence of species admixtures or microbial contaminants. For the financial gain or easy availability, the manufacturers may have added closely related species along with the original species resulting into the additional bands. These possibilities should be addressed by pharmacies or healthcare authorities before the Triphalachurna formulations are launched in the market for public consumption.

5. CONCLUSION

From the above studies, we can conclude that the presented DNA barcoding assisted standardization approach can be successfully employed to authenticate the polyherbal formulation Triphala. The designed primers were found to be capable for authentication of all the three species i.e. *Terminalia bellirica, Terminalia chebula*and*Phyllanthusemblica*before they are blended together into Triphala formulation.Additionally, the designed primers successfully ensured that the correct component plant species were incorporated in the commercially available, processed Triphalachurna samples included the correct component species. Thus, DNA barcoding can be used as a standalone technique for species authentication or can be used along with traditional standardization approaches such as morphological, macroscopic, biochemical profiling to develop a comprehensive approach for quality assurance of ayurvedicpolyherbal formulations.

COMPETING INTERESTS

Compliance with Ethical standards:

The authors declare that they have no conflict of interest (Financial/Non-financial).

No human participants/animals were involved in the present study.

No funding was received to carry out this research work.

Conflict of interest statement:

The authors declare that they have no conflict of interest.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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