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RESEARCH

# Overcoming Challenges in DNA Extractions from Triphala Ingredients: A Way Forward for Optimization of Conventional and Digital PCR Assays for Molecular Authentication

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## Abstract

*Terminalia bellirica* (TB), *Terminalia chebula* (TC), and *Phyllanthus emblica* (PE) fruits are renowned for their diverse therapeutic benefits, propelling their cultivation and use in herbal remedies. However, the global surge in demand driven by the awareness and long-term benefits of using herbal medicines has inadvertently led to a rise in adulteration practices within the herbal market. Recent advancement in DNA authentication of herbal products is constrained by poor quality and quantity of PCR amplifiable DNA obtained from the dried and polyphenol-rich fruits of processed herbal products, resulting in inconsistent PCR amplification due to heterogeneous secondary metabolites. This study tailored a DNA isolation protocol by optimizing buffering strength to stabilize pH and adding phenolic compound scavenger additives, such as polyvinylpyrrolidone, during the cell lysis step. The implemented procedure resulted in significant enhancements in both the quantity and quality of PCR amplifiable DNA. PCR amenability was evaluated using *ITS2* metabarcode. Later, species-specific assays, targeting ITS-based SCAR markers specific to TB, TC, and PE, were performed on six market powders for each plant species. TB, TC, and PE were detected in 100, 83.3, and 50% of the six market samples, respectively. Digital PCR increases the assay's sensitivity by two-fold compared to conventional PCR. To the best of our knowledge, this is the first instance of utilizing dPCR for authenticating TB, TC, and PE fruits. The improvised DNA extraction protocol successfully demonstrates how a comprehensive analysis of PCR amplifiable DNA isolation and PCR dynamics enables the effective resolution of challenges related to poor DNA quality and quantity, as well as the inconsistency encountered during PCR due to the heterogeneity of polyphenols.

**Keywords** Digital PCR · Improvised PCR amplifiable DNA extraction protocol · PCR · Species-specific PCR assay · *T. bellirica* · *T. chebula* · *P. emblica*

## Introduction

*Terminalia bellirica* (family: Combretaceae; TB), *Terminalia chebula* (family: Combretaceae; TC), and *Phyllanthus emblica* (family: Euphorbiaceae; PE) are among the top 70 listed highly traded medicinal plant species from tropical forests. In 2014–2015, approximately 14,200, 2700, and 6000 MT consumption had been reported, respectively, for each species (Goraya and Ved 2017). TB is characterized by its richness in glucoside, gallotannic acid, ellagic acid, lignans, flavones, and various other compounds, which contribute to its antimicrobial, antioxidant, hepatoprotective, and bronchodilatory properties (Deb et al. 2016). TC, on the other hand, is known for its phenolics and tannins, offering antioxidant, anti-inflammatory, antidiabetic, and wound-healing effects (Cock 2015; Sultan et al. 2023). PE contains vitamin C, amino acids, and phenolic compounds providing antioxidant, anti-inflammatory, and hepatoprotective benefits (Gaire and Subedi 2014). These three plants are used as a single drug or as polyherbal formulation as Triphala Rasayana. It stimulates longevity and rejuvenation.

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It is multi-therapeutic and used in treating digestive problems such as ulcers. Moreover, it is anti-inflammatory, cardioprotection, and neuroprotectant and has antioxidant and immunostimulatory effects (Peterson et al. 2017).

The incidence of unintentional and economically motivated adulteration is increasing due to a globalized economy, industrialization, and e-marketing (Raclariu et al. 2018; Ichim 2019). With this, the authentication of herbal products has become a burning concern in consumer trust and acquisition (Ichim 2019). To address the growing concerns of unintentional and economically motivated adulteration, various pharmacopeia, including the Chinese Pharmacopoeia, United States Pharmacopeia, British Pharmacopoeia, Japanese Pharmacopoeia, and Hong Kong Chinese Materia Medica, have advocated DNA-based authentication, as it has proven universal, accurate, robust, and cost-effective in compare to analytical methods (Wu and Shaw 2022). However, one of the significant limitations in DNA-based authentication is extracting high-quality PCR amplifiable DNA in sufficient quantities from medicinal botanicals or herbal products (Parveen et al. 2016).

Handling dried tissues containing high levels of secondary metabolites like polyphenols and polysaccharides poses a greater challenge (Singh and Bandana 1999; Sharma et al. 2017). During DNA extraction, when the cells are lysed, polyphenols are released from vacuoles and readily undergo oxidation reaction (Varma et al. 2007; Ghadia et al. 2016; Schenk et al. 2023). The formation of irreversible bonds between oxidized polyphenols and DNA poses a significant concern. Consequently, it hinders downstream processing like PCR amplification and leads to potential false-negative and heterogeneous results (Sharma et al. 2017; Heikrujam et al. 2020). The primary challenge lies in obtaining high-quality, PCR-amplifiable DNA from processed final herbal products to develop DNA-based authentication processes (Sharma et al. 2017; Raclariu et al. 2018; Ichim 2019; Wu and Shaw 2022). *ITS2* emerged as the preferred DNA marker for authenticating species within the Indian *Terminalia* genus (Nithaniyal and Parani 2016). Concurrently, in the authentication of Thai *Terminalia* crude drugs, methodologies such as PCR–RFLP and ARMS-PCR were employed (Intharuksa et al. 2016). Authentication methods for the *Phyllanthus* genus primarily depended on RAPD-SCAR or ITS-SCAR-based approaches (Dnyaneshwar et al. 2006). These techniques demonstrate a precise and scientific approach to accurately identifying species within the respective plant genera using DNA extracted from leaf tissues.

In light of these challenges, this study aims to develop a DNA-based method for authentication of polyphenol-rich dried fruits used as herbal therapeutics. For this purpose, a protocol was improvised to obtain PCR amplifiable DNA from the dried fruits of TB, TC, and PE by tailoring the isolation protocol with increasing buffering strength and polyvinylpyrrolidone (PVP) concentration. The effectiveness of extracting PCR-amplifiable DNA was tested using species-specific PCR and dPCR assay. The applicability of the complete method was tested on TB (Baheda), TC (Harde), and PE (Amala) herbal products. Our goal was to improve the precision and trustworthiness of PCR results by reducing variability caused by the heterogeneity of secondary metabolites present in the extracted DNA. The aim was achieved by improvising PCR amplifiable DNA isolation in polyphenol-rich dried fruits. The complete approach developed in the present study will open the avenue for DNA-based authentication methods for complex medicinal materials rich in polyphenols.

## Material and Methods

### Reference Plant Material Collection, DNA Isolation, and rbcL Gene Sequencing

Leaves and fruits of the *Terminalia bellirica* (TB), *Terminalia chebula* (TC), and *Phyllanthus emblica* (PE) were collected from the Directorate of Medicinal and Aromatic Plants Research (DMAPR), Anand (Gujarat, India), with the aid of a taxonomist. Herbarium voucher specimens were developed for all three plants and submitted to the institutional herbarium with the following specimen IDs: TB = BG-201130–0011; TC = BG -201,130–0012: PE = BG-201130–0013. Leaf collection aimed to assess the sensitivity of PCR assays with comparison to polyphenol-rich dried fruit tissue samples obtained from the same plant. DNA isolation from the leaves was performed using the DNeasy plant mini kit (Qiagen, India) following the manufacturer's instructions. Molecular authentication of reference plants was done using *rbcL* (Maloukh et al. 2017) gene sequencing (Table 1). Correct species assignment was obtained in the BLAST results, and sequences have been submitted to the NCBI database with accession numbers MW628925, MW628910, and MW628927 for TB, TC, and PE, respectively. Further, six dried fruit powders of TB, TC, and PE were also collected from the local market labeled as Baheda (B1 to B6), Harde (H1 to H6), and Amla churna (A1 to A6), respectively, from Ahmedabad, Gujarat, India.

### DNA Extraction Protocol from Dried Fruits and Market Products

Eleven different DNA isolation protocols with minor but significant modifications were executed (Table 2) with the powder prepared from the dried fruit of TB, TC, and PE. The first attempt was done using the DNeasy Plant Mini Kit (QIAGEN, India). Protocols 2 and 3 were used here with

**Table 1** Sequences of species-specific primers and universal primers with their annealing temperature

| Plant species | Primer | Sequence (5′ to 3′) | Annealing temperature | Amplicon length | References |
|---|---|---|---|---|---|
| *T. bellirica* L | TB—forward | GTCGATCTAAGCCCCAGCAG | 56 °C | 150 bp | (Sharma and Shrivastava, 2016) |
| *T. chebula* L | TC—forward | GATGGGAGGATGGTCCGGGA | | 244 bp | |
| *ITS* 4 | ITS 4—reverse | TCCTCCGCTTATTGATATGC | | | |
| *P. emblica* L | PE—forward | TTTAGTCACTGCGGATGGTG | | 212 bp | (Bandyopadhyay and Raychaudhuri, 2010) |
| | PE—reverse | GAGATATCCGTTGCCGAGAG | | | |
| *rbcL* | rbcLa—forward | ATGTCACCACAAACAGAGACTAAAGC | 60 °C | 600 bp | (Maloukh et al. 2017) |
| | rbcLa—reverse | GTAAAATCAAGTCCACCRCG | | | |
| *ITS2* metabarcode | *ITS2*—forward | CRRAATCCCGTGAACCATCGAGTCYT | | 310–330 bp | (Travadi et al. 2023) |
| | *ITS2*—reverse | AGCGGGTRRTCCCRCCTGACYTG | | | |

minor modifications as described in (Warude et al. 2003). In both protocols, modified CTAB buffer was used with an additional 100 mg PVP powder as described in Table 2. The only difference between protocols 2 and 3 is after PCI treatment (Phase separation) protocol 2 followed the isopropanol precipitation, while protocol 3 was coupled with a column-based purification procedure (Table 2). Later, in protocols 4 and 5, the buffer strength was increased from 0.1 M to 0.2 and 0.5 M Tric-Cl in the lysis buffer composition to normalize the pH. Additional phenol treatment was applied in protocol 6 to remove polyphenol-PVP and CTAB-polysaccharide complex during phase separation. Socking-out procedures were also used to soften the tissue with a one-hour or overnight incubation at room temperature in protocols 7 and 8, respectively. Finally, during the grinding step in protocols 9, 10, and 11, different PVP concentrations of 200, 500, and 1000 mg were introduced (Table 2). These many variations allowed us to explore a range of modifications to achieve effective DNA isolation from TB, TC, and PE dried fruits. The protocol that gives the highest DNA yield as well as PCR amplifiability, i.e., protocol 11, is described in detail below.

A hundred milligrams of fruit/powder and 1 g of PVP was minced with liquid nitrogen, and then 3 mL of optimized extraction buffer [0.5 M Tris–Cl (pH 8.0), 20 mM EDTA (pH 8.0), 1.5 M NaCl, 4.0% CTAB (w/v), 5% β-mercaptoethanol (v/v), and 2% PVP (w/v)] was added. The mixture was incubated at 65 °C for 30 min in the water bath with shaking at intervals of 10 min. After that, 1 mL of phenol was added to the supernatant lysate and mixed thoroughly, followed by centrifugation at 15,000X g for 10 min at 4 °C. The aqueous phase was collected into a fresh vial, and 1 mL of phenol to chloroform to isoamyl alcohol (25:24:1) was added to the supernatant, then centrifuged at 15,000X g for 5 min at 4 °C. These steps were repeated. Then, the DNA was purified from the aqueous phase using a silica column by following the DNeasy Plant Mini Kit (QIAGEN, India) protocol from the binding step. DNA from the fruits was quantified using the 1X HS and Qubit fluorometer 4.0 (Thermo Fisher Scientific, USA).

## DNA Quality Evaluation Using *ITS2* Metabarcode

To evaluate the quality of the extracted DNA from three different fruits and market samples, the *ITS2* metabarcode primers and thermal cycler conditions as described in our previous study by Travadi et al. (Travadi et al. 2023). The rationale behind choosing the *ITS2* region was its ability to produce a 300-base pair amplicon size, facilitating the evaluation of sheared DNA extracted from fruit tissues. Furthermore, all species-specific primers, designed from the *ITS*-SCAR region, having amplicon lengths below 250 base pairs.

## Species-Specific PCR Assay

For the species-specific assay, primers for TB and TC were obtained from Sharma and Shrivastava (2016), while PE primers were obtained from Bandyopadhyay et al. (2010) (Table 1). PCR conditions used as follows: A total of 20 µL of reaction mixture contained 10.0 µL of Emerald Takara master mix (2X), 2.0 µL of DNA (5 ng/µL), 1.0 µL of each forward and reverse primer (5.0 pmol), 2.0 µL of BSA (2.0 mg/mL), and 4.0 µL of PCR grade water. The thermal cycler conditions were optimized as follows: Initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. At the end of 35 cycles, a final extension at 72 °C for 5 min was performed. The sensitivity assay was also carried out with TB, TC,

**Table 2** Improvisation steps in DNA isolation procedures and comprehensive analysis of DNA quantification (D), *ITS2* PCR (IP) amplification, and species-specific PCR (SP) amplification for fruits of *T. bellirica* (TB), *T. chebula* (TC), and *P. emblica* (PE) with 11 protocols

| DNA isolation | | | | | | | | | | | | Comparative analysis of DNA quantification (D), ITS2 PCR (IP) amplification, and species-specific PCR (SP) amplification | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Protocol number | Tissue socking procedure (h) | Lysis procedure | | | | | | | Phase separation/cleaning procedure | | DNA purification | | | | | | | | | |
| | | CTAB buffer composition | | | | | | PVP during the grinding (mg) | Phenol treatment | PCI[$] treatment | Column purification (c)/ isopropanol precipitation (I) | TB | | | TC | | | PE | | |
| | | CTAB (%) | Tric-Cl (M) | EDTA (mM) | NaCl (M) | PVP (%) | BME (%) | | | | | D | IP | SP | D | IP | SP | D | IP | SP |
| 1 | No | DNeasy plant mini kit (QIAGEN, India) | | | | | | | | | | *TL | + | + | *TL | − | − | *TL | − | − |
| 2 | | 4 | 0.1 | 20 | 1.4 | 2 | 5 | 100 | No | Yes | I | 0.32 | + | + | 0.04 | − | − | 0.28 | − | − |
| 3 | | | 0.1 | | | | | | | | C | 0.68 | + | + | 0.12 | − | − | 0.32 | − | − |
| 4 | | | 0.2 | | | | | | | | | 3.64 | + | + | 0.20 | − | − | 0.44 | − | − |
| 5 | | | 0.5 | | | | | | Yes | | | 9.84 | + | + | 0.79 | − | − | 1.04 | + | + |
| 6 | | | | | | | | | | | | 10.00 | + | + | 1.28 | − | − | 1.52 | + | + |
| 7 | 1 | | | | | | | | | | | 9.36 | + | + | 0.86 | − | − | 2.68 | + | + |
| 8 | 24 | | | | | | | | | | | 10.84 | + | + | 0.79 | − | − | 4.40 | + | + |
| 9 | No | | | | | | | 200 | | | | 11.56 | + | + | 3.74 | + | + | 5.68 | + | + |
| 10 | | | | | | | | 500 | | | | 13.84 | + | + | 5.60 | + | + | 7.44 | + | + |
| 11 | | | | | | | | 1000 | | | | 18.00 | + | + | 12.00 | + | + | 9.32 | + | + |

**TL* too low, *PCI*[$] phenol chloroform isoamyl alcohol, *D* mean ($n=2$) of fruit DNA quantification (µg/g)

and PE primer with respective fruit DNA with 10.0, 5.0, 2.5, 1.25, 0.6, 0.3, 0.15, 0.07, and 0.01 ng of DNA input. The aforementioned protocols were performed for the respective species-specific primer sets for the sensitivity assay with both leaf and fruit DNA and also with market formulations.

### Digital PCR (dPCR)

The dPCR (Digital PCR) reaction mixture was constituted with 13.7 µL of Evagreen master mix (2X) from QIAcuity EG PCR Kit (Qiagen, India), 1.0 µL of forward and reverse primers (10 pmol each), 2 µL of DNA template, 1 µL of *Eco*R1 (1U/µL), and 20.1 µL of nuclease-free water was added in a total of 40 µL volume. The reaction was incubated for 10 min at room temperature for DNA digestion and then loaded to a QIAcuity Nanoplate 26 k 24-well plate. The plate was run in QIAcuity Digital PCR System, Qiagen, India. The thermal cycling conditions are as follows; initial denaturation at 95 °C for 2 min, then 40 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 40 s, and a final cooling down at 40 °C for 5 min. The PCR amplification signal was determined using the green channel total copy numbers of target DNA was calculated using the following formula: [(total PCR reaction volume/µL of DNA template used in PCR reaction) × obtained copy numbers/µL] as per the guideline given in the QIAcuity® user manual extension. The sensitivity assay was also carried out with TB, TC, and PE primer with respective fruit DNA with 10.0, 5.0, 2.5, 1.25, 0.6, 0.3, 0.15, and 0.07 ng of DNA input.

## Results and Discussion

High quality and quantity of DNA isolation from medicinal botanicals have been notorious due to the coprecipitation of DNA with polyphenols and polysaccharides. Due to the inherent biochemical heterogeneity, this often results in inconsistent or unsuccessful PCR amplification (Uncu et al. 2018). Moreover, plant biochemical heterogeneity is highly influenced by geographical and environmental factors, processing of the plant tissue, age and tissue type, and season variation. These factors significantly impact the DNA isolation procedure as well as the quality and quantity of the obtained DNA. Henceforth, major and minor modifications of the CTAB (cetyltrimethylammonium bromide) (Doyle and Doyle 1987) isolation protocol demonstrated that by tailoring DNA isolation protocol, high quality and quantity of PCR amplifiable DNA from different plant species and tissue having heterogeneous chemical constituents could be achieved (Varma et al. 2007; Schenk et al. 2023).

### Improvisation of PCR Amplifiable DNA Isolation form TB, TC, and PE Fruit Powder Using Additives

*Terminalia bellirica* (TB), *Terminalia chebula* (TC), and *Phyllanthus emblica* (PE) fruits are of potent multi-therapeutic ayurvedic formulations with high cultivation and consumption economics (Goraya and Ved 2017). Various pharmacopeia have included DNA-based authentication approaches, such as barcoding and species-specific PCR assay to authenticate raw herbal products. It is proven cost-effective, universal, and robust compared to the present chemical analytical methods(Raclariu et al. 2018; Wu and Shaw 2022). However, low quality and quantity of the PCR amplifiable DNA are the major limitation to authenticate raw material. Apart from this, there is no universal DNA isolation protocol for all plant species and their different tissue types. Researchers are facing challenges in obtaining PCR amplifiable DNA. Henceforth, the objective of the present study was to develop a protocol to obtain homogeneous PCR amplifiable DNA from the polyphenolic-rich fruit to strengthen the results of the DNA-based authentication approach.

DNA isolation from dried fruits of TB, TC, and PE was optimized using minor and major modifications using CTAB and/or coupled with DNeasy plant mini kit (Qiagen, India). Initially, DNA isolation was done using the DNeasy plant mini kit (Qiagen, India) following the manufacturer's instructions for all three fruits powder; however, low DNA yield was obtained for all three fruits. Notably, *ITS2* metabarcode gave positive amplification only for the TB fruit (Table 2; Fig. S1a).

During the cell lysis step of DNA extraction, DNA readily reacts with polyphenols and polysaccharides, forming covalent interactions and co-precipitation with DNA. The incorporation of polyvinylpyrrolidone (PVP) during tissue maceration has been documented to effectively scavenge polyphenols during the tissue lysis (Porebski et al. 1997). Additionally, a higher concentration of cetyltrimethylammonium bromide (CTAB) was found to be effective against polysaccharides. Therefore, a 4% CTAB concentration was implemented in this study. In 2003, Warude et al. (2003) developed a CTAB manual protocol for isolating DNA from acidic dry fruits of TB, TC, and PE. Henceforth, with minor modifications in the CTAB extraction buffer and an additional 100 mg PVP against 100 mg powder sample, the cell lysis using grinding was accomplished, followed by PCI and CIA aqueous phase separation. The downstream processing was performed by isopropanol precipitation in protocol 2, while protocol 3 was coupled with column binding, as mentioned in Table 2. During the lysis phase, a rise in pH was observed may be due to oxidative reactions, which was normalized intermittently during the incubation time as mentioned in (Warude et al. 2003). However, no significant

improvement in DNA concentration and PCR amplification was observed in the TC and PE fruit case, with either of the protocols (Table 2; Fig. S1b, S1c).

In response to the observed pH fluctuations impacting DNA stability, efforts were made to address potential DNA degradation at low pH. This involved adjusting the buffering strength of Tris–Cl in the CTAB lysis buffer to 0.2 M in protocol 4 and 0.5 M in protocol 5. When 0.5 M of Tris–Cl buffering strength was introduced, it was able to stabilize acidic pH rise observed during lysis for all three fruits, and that led to an increase in DNA concentration with 9.8, 0.7 and 1.0 µg/g for TB, TC, and PE fruit tissue (Table 2). *ITS2* metabarcode amplification results revealed that PCR amplifiability was observed in PE and TB fruit DNA (Fig. S1a, S1c), but no amplification was observed in the case of TC fruit DNA.

After incubation in all aforementioned protocols, a notably viscous lysate was observed, particularly in PE and TC lysates. This viscosity could likely to be attributed to the formation of complexes between PVP-polyphenol and CTAB-polysaccharide, which were not completely eliminated during the PCI and CIA phase separation, consequently impeding downstream processing. To address this issue, additional phenol treatments were introduced before the PCI treatment. This modification resulted in the observation of upper aqueous, middle solid and cloudy, and lower organic phases, in which middle phase effectively able to eliminate PVP-polyphenol and CTAB-polysaccharide complexes (Doyle and Doyle 1987; Lodhi et al. 1994; Porebski et al. 1997). With the implementation of this modified protocol, there was an increase in DNA concentration for all three types of fruit DNA. However, TC fruit DNA did not exhibit successful amplification with the *ITS2* minibarcode (Table 2; Fig. S1b).

During the grinding process, it was noted that the PE dry fruit tissue was toughest to maceration compared to TB and TC fruits. To address this, a soaking-out technique was employed for the softening of the tissue through two protocols (7 and 8) before DNA extraction from TB, TC, and PE dried fruits (Singh and Bandana 1999; Asish et al. 2010). Protocol 7 involved a 1-h incubation, while protocol 8 entailed an overnight incubation at room temperature. The results revealed an increased PE fruit DNA concentration, 2.68 and 4.4 µg/g for protocols 7 and 8, respectively (Table 2). That is likely attributed to improved tissue softening and enhanced homogenization during grinding. However, it's noteworthy that the *ITS2* metabarcode failed to amplify TC fruit DNA from protocols 7 and 8 (Fig. S1b).

The pronounced browning was observed in TC fruit DNA during the elution step in protocols 1–8, suggests a co-precipitation polyphenols along with DNA. This interaction has the potential to hinder the efficiency of PCR amplification, indicating a possible impediment to the amplification process (Porebski et al. 1997; Santos et al. 2023). PVP plays a pivotal role in quenching the interaction between oxidized phenol and DNA, favoring interactions with oxidized phenol instead. Henceforth, various high amounts of PVP (200, 500, and 1000 mg) were introduced during the grinding and cell lysis step.This resulted in a substantial improvement in DNA concentration, resulting in 18 µg/g for TB, 12 µg/g for TC, and 9.32 µg/g for PE fruits with protocol 11 and given positive amplification with *ITS2* metabarcode for all three fruits DNA (Table 2; Fig. S1a, S1b, S1c). A charcoal-based DNA isolation method from mature leaves, embryos and bark of *T. bellerica*, the bark of *T. chebula* was developed by Sharma et al. (2017). DNA extracted from TB, TC and PE fruits by (Warude et al. 2003) was showing PCR amenabilities for various polymorphic makers. However, we could not able to amplified DNA trough PCR with *ITS* primers (Table 1) from fruits of TB, TC, and PE using Protocol ID 1 which might be due to highly acidic pH, high polyphenolic and polysaccharide content and secondary metabolites content of fruits compare to leaves and bark. Therefore, here, we modified DNA isolation protocols for all three fruits by using modified CTAB buffer composition and adding additional PVP. On the whole, our results revealed that PCR amplifiable DNA was hardest to achieve in TC fruits due to high polyphenols present in the species and its fruit tissue. The polyphenol readily reacts with DNA and was responsible for the browning effect and degradation of DNA due to acidic pH. The use of high amount of PVP during the grinding scavenges the polyphenols and high molarity of the Tric-Cl buffering strength stabilize the pH, and able to maintain the pH, while for the PE, the real challenge was high tannin and polysaccharide content present in fruit tissue.

## Species-Specific PCR Assay for TB, TC and PE Fruit Powder

The species-specific PCR assay conditions were optimized using the respective leaves DNA of TB, TC, and PE. The results revealed that 56 °C is the optimal temperature for the primer annealing (data not shown). DNA isolated from fruits of TB, TC, and PE using 11 protocols was subjected to optimized species-specific PCR assay. The DNA extracted from TB fruits using all 11 protocols was successfully amplified using species-specific assay (Fig. 1a), while in the case of the TC, amplification was observed only with three protocols, i.e., protocol number 9 to 11 (Fig. 1b). Protocol number 5 to 11 gave positive amplification for the PE DNA (Fig. 1c). Similar concordance was observed during the assessment of quality evaluation with *ITS2* minibarcode for TB, TC, and PE fruits. The study observed that increasing the buffering strength and phenol treatment resulted in enhanced PCR band intensity for TB and PE fruit DNA. However, various

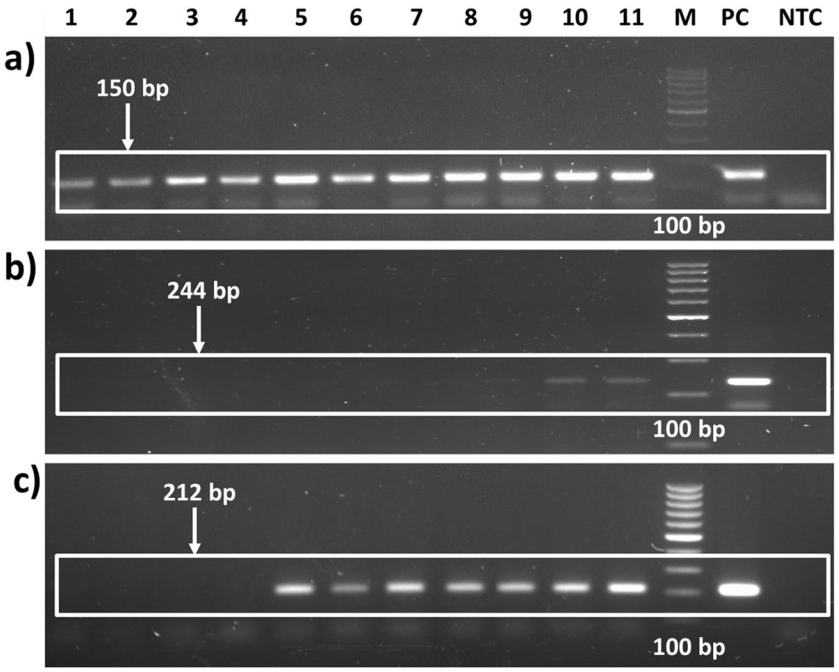


**Fig. 1** Species-specific PCR assay with 11 different protocols of DNA isolation from fruits. **a** *Terminalia bellirica* (TB), **b** *Terminalia chebula* (*TC*), and **c** *Phyllanthus emblica* (*PE*); 1–11 = Protocol ID; PC = DNA isolated from respective leaves; NTC = no template control; M = 100 bp DNA ladder (100–1000 bp). The amplicon size for the TB-, TC and PE-specific primers is 150, 244, and 212 bp, respectively

concentrations of PVP and leaching-out procedures did not affect the band intensity of PCR amplification in the case of TB and PE-specific assays. For TC fruit DNA, notably good band intensity was observed with high PVP concentrations of 500 and 1000 mg (Fig. 1b). The results highlighted the high PVP amount used during grinding procedure able to reduce browning of DNA elute and facilitating PCR amplification in TC fruit DNA.

A sensitivity assay was employed to determine the minimum DNA concentration required for positive amplification determination on an agarose gel. This assay aimed to assess the dynamics of the PCR in the presence of secondary metabolites and sheared DNA extracted from dried fruit tissue. The sensitivity assay was conducted in comparison to leaf tissue, providing a reference for the primer performance under these conditions. (Fig. 2a–f). For this purpose, DNA concentration in the range of 0.01 ng to 10 was used, while using DNA extracted from the leaves, 0.01 ng DNA was found to be sufficient to see band on gel for all plants. Similarly, in the case of the fruits, the results revealed that

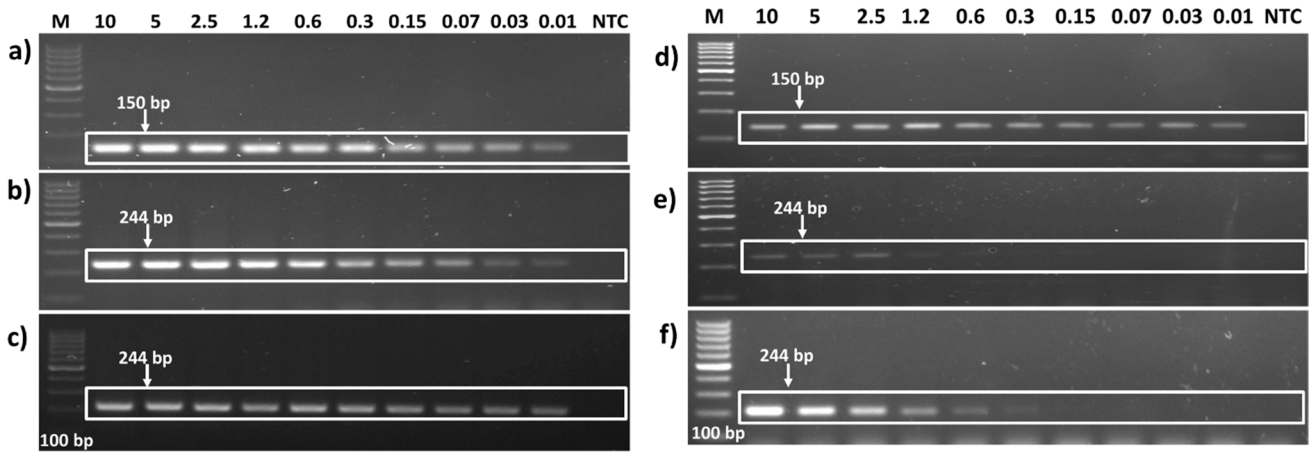


**Fig. 2** Sensitivity assay with 10 ng to 0.01 ng DNA concentration using DNA extracted from leaves and dried fruit tissues with species-specific primers. **a, b, c** *Terminalia bellirica* (TB), *Terminalia chebula* (TC), and *Phyllanthus emblica* (PE) for the DNA extracted from the leaves of the respective plant and **d, e, f** for DNA extracted from the dried fruit of the respective plant. NTC = no template control; M = 100 bp DNA ladder (100–1000 bp). The amplicon size for the TB-, TC-, and PE-specific primers is 150, 244, and 212 bp, respectively

0.01 ng DNA input give amplification for TB (Fig. 2d), while, in the case of TC and PE fruits DNA, 0.15 ng of minimal DNA concentration was required to observe amplification on agarose gel (Fig. 2e, f). Hence, the minimum DNA concentration needed for successful amplification was observed to be 10 times higher when using DNA extracted from the fruits of TC and PE compared to their respective leaf DNA. (Fig. 2b, c, e, f). This clearly depicts the impact of different tissues and their chemical heterogeneity able to hinder DNA and PCR dynamics. However, the length of the amplicon is directly proportional to the success rate of PCR amplification in cases of low-quality and low-quantity DNA (Särkinen et al. 2012; Techen et al. 2014). Notably, the TB primer (amplicon length 150 bp) generated significantly shorter amplicons compared to the TC (244 bp) and PE primers (212 bp), which might be another major factor contributing to PCR dynamics.

Further, to check the applicability of our improved DNA extraction procedure, we procured six market formulations of each of TB, TC, and PE labeled as Baheda (TB fruit), Harde (TC fruit), and Amla (PE fruit) from the local market. Amplification using *ITS2* metabarcode and species-specific primers with aforementioned PCR conditions were carried out. Successful PCR amplification was observed with all the TB fruit (Baheda) powder products (Fig. 3a). In the case of TC (Harde) powder products, positive amplification was observed in five samples (Fig. 3b), whereas only three samples exhibited positive amplification for PE (Amla) powder products (Fig. 3c). Nevertheless, all fruit powders exhibited positive amplification with the *ITS2* minibarcode (Fig. S2). This observation suggests that the absence of the desired species-specific amplicons in these samples may be attributed to the substitution of the original species with the spurious one or complete missing of the same. In our previous study, only 57.7% of Tulsi (*Ocimum tenuiflorum*) powder had the presence of the target species (Travadi et al. 2022b), and only in 22.2% of Bramhi (*Bacopa monnieri*/*Centella asiatica*) powder we were able to detect either *B. monnieri* or *C. asiatica* using species-specific PCR assay(Shah et al. 2023). The DNA isolation process for Tulsi and Brahmi powder is simpler compared to that of TB, TC, and PE fruit powder because they contain leaf tissue as ingredients, rather than fruits. This relative simplicity can be attributed to the inherent differences in tissue types and the composition of biochemical constituents in these plant materials. A sensitive and robust DNA extraction method for authenticity determination of dried roots of *Glehnia littoralis* was developed, which was able to detect 0.1% intentional adulterant with 0.01 ng limit of detection (Lin et al. 2023). Notably, PCR dynamics could be affected by poor DNA quality arising from variations in tissue age, storage, processing conditions, and even inter-sample variability (Sharma et al. 2017). Here, we successfully demonstrated that by tailoring the DNA isolation protocol using additives that enabled successful species-specific PCR amplification for dried fruits with high polyphenol content, reducing inconsistencies stemming from secondary metabolite heterogeneity.

## Determination of the Dynamic Range of Digital PCR with Fruit DNA

A dPCR assay assessed the dynamic range and its potential in authenticating polyphenol-rich fruits of TB, TC and PE DNA. In the dPCR, DNA gets diluted along with secondary metabolites, enhancing precision, accuracy, and robustness. In a preliminary experiment of dPCR optimization, the optimal temperature was found to be 56 °C for all species-specific primers. DNA from fruits of TB, TC, and PE was subjected to a two-fold dilution, ranging from 10 to 0.07 ng, and dPCR assay was carried out in replicates. The resulting mean copy numbers ranging from 1,71,674 to 2328 for TB fruit DNA, 1230 to 25 for TC fruit DNA, and 1905 to 16 for PE fruit DNA were obtained against 10 to 0.07 ng DNA input (Table S1). The results indicated the positive correlation of increasing DNA input with positive valid partitions (Fig. 4a–c). The linear regression correlation coefficient between DNA concentration and copy numbers was obtained to assess the dynamic range, for the DNA

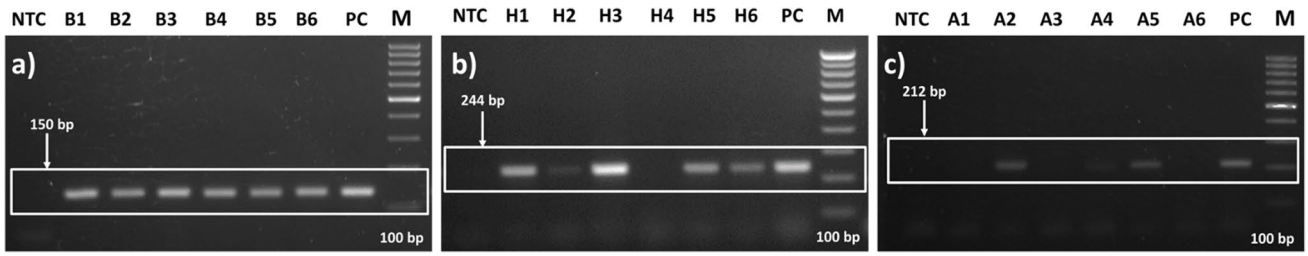


**Fig. 3** Species-specific PCR assay with single drug market formulation with respective primers. **a** *Terminalia bellirica* (TB), B1 to B6 TB (Baheda) fruit powder, **b** *Terminalia chebula* (TC), H1 to H6 TC (Harde) fruit powder, and **c** *Phyllanthus emblica* (PE), A1 to A6 PE (Amla) fruit powder. PC = DNA isolated from respective dried fruit tissue; NTC = no template control; M 100 bp DNA ladder (100–1000 bp) The amplicon size for the TB-, TC-, and PE-specific primers is 150, 244, and 212 bp, respectively

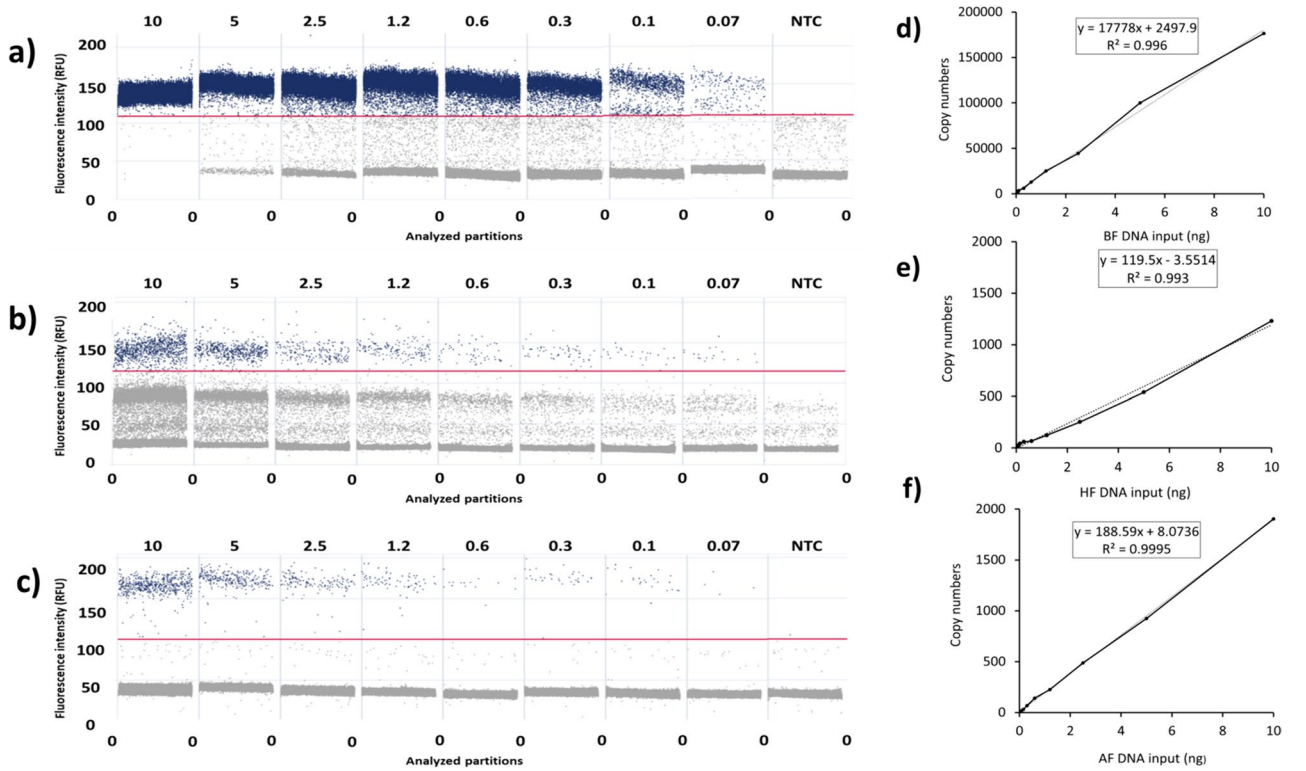


**Fig. 4** Digital PCR assay with *Terminalia bellirica* (TB), *Terminalia chebula* (TC) and *Phyllanthus emblica* (PE) fruit DNA. The red line represents the fluorescence amplitude threshold. Positive partitions were observed above the threshold and negative partitions were below the threshold. NTC = no template control. Digital PCR assay with **a** TB fruit DNA, **b** TC fruit DNA, and **c** PE fruit DNA. **e, f, g** The linear relationship between DNA concentration (ng) extracted from TB, TC, and PE fruit samples and the corresponding DNA copy numbers. The equation provided within each figure represents the linear regression equation, where "*y*" represents copy numbers, "*x*" signifies input target DNA concentration, and "$R^2$" represents the coefficient of determination

extracted from the fruits of TB, TC, and PE. The obtained results depicted $R^2 > 0.99$ for all three fruits; henceforth, we found 10 ng to 0.07 ng optimal dynamic range for all three fruits of TB, TC, and PE, as illustrated in (Fig. 4d–f). This range signifies the concentration levels within which our analysis consistently exhibited high accuracy and linearity. For the TC and PE fruit DNA, the sensitivity was increased two fold than conventional PCR. However, dPCR dynamics will be highly influenced by various factors such as gene target, copy number, DNA quality, and quantity. In our previous study, dPCR gives up to 0.001 ng sensitivity for DNA obtained *O. sanctum* and *O. basilicum* (Travadi et al. 2022b), while for 0.06 ng DNA of sensitivity was observed for DNA of *P. nigrum* and *C. papaya* (Travadi et al. 2022a). Similar studies have been also reported in other plants by Yu et al. (2022) (Yu et al. 2022) where authors developed a dPCR assay for the authentication of dried root of *Panax notoginseng* powder, which gives good linearity within the range of 5–45 ng/µL. The overall results indicate that improvised DNA isolation protocol for the dried fruit powder significantly improvised the quality of DNA, which is reflected upon the optimal dynamic range (10 to 0.07 ng of DNA) and 0.07 ng sensitivity. In conclusion, dPCR can be a potential tool in DNA-based authentication when inconsistency was observed in conventional PCR due to heterogeneity of secondary metabolites, sheared DNA, and lower yield.

## Conclusion

DNA-based authentication of herbal products is a universal, robust, accurate, and cost-effective approach. However, challenges like extracting PCR-amplifiable DNA and inconsistent or false negative results due to secondary metabolite interference, remain significant limitations. In the present investigation, PCR amplifiable DNA has been obtained from the challenging polyphenol-rich dried fruits like TB, TC, and PE. Different parameters, modified in the present protocol such as enhanced buffering strength with Tris–Cl, increased PVP concentration, and combining manual and column-based protocols, we achieved PCR-amplifiable DNA with sufficient yield. Exploring additional additives and their roles in DNA isolation is still promising. The protocol developed in this study and the results

obtained in PCR with species-specific assay revealed their applicability to market formulation. Further, digital PCR is found to excel in sensitivity, robustness, and accuracy, while conventional PCR can be considered a more cost-effective method.

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**Author Contribution** TT: molecular biology experiments, data analysis, writing, and validation of manuscript; SS: manuscript review and editing; RP: manuscript review and editing; CJ: project administration, methodology, supervision, and review and editing; PJ: conceptualization, methodology, supervision, and review and editing; MJ: principal investigator, conceptualization, methodology, supervision, and review and editing. All authors reviewed the manuscript.

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**Data Availability** All analyzed data generated in this study are included in this article and its supplementary information file.

## Declarations

**Competing interests** The authors declare no competing interests.

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