Chapter 2

Optimization of DNA Isolation from Fruits of *Terminalia bellirica* (TB), *Terminalia chebula* (TC), and *Phyllanthus emblica* (PE) for PCR-Based Authentication

2.1 Introduction

Terminalia bellirica (TB), *Terminalia chebula* (TC), and *Phyllanthus emblica* (PE) are valued medicinal plants, used individually and in the polyherbal formulation Triphala Rasayana, due to their diverse therapeutic applications and economic importance. DNA-based authentication offers a universal, accurate, robust, and cost-effective method for addressing adulteration in botanicals (Wu & Shaw, 2022). However, obtaining high-quality, amplifiable DNA, especially from processed herbal products, presents a significant challenge (Ichim & Booker, 2021; Parveen et al., 2016; Raclariu et al., 2018; Sharma et al., 2017; Wu & Shaw, 2022). This is particularly true for TB, TC and PE, due to the co-precipitation of DNA with polyphenols and polysaccharides during extraction (Uncu et al., 2018). Plant biochemical heterogeneity, influenced by factors like geography, environment, processing, age, tissue type, and seasonality, further complicates DNA isolation (Sharma et al., 2017, Singh et al., 1999).

Upon cell lysis, polyphenols are released and oxidized, forming irreversible bonds with DNA (Ghadia et al., 2016, Heikrujam et al., 2020, Schenk et al., 2023, Sharma et al., 2017, Varma et al., 2007). This hinders downstream processes like PCR amplification, potentially leading to false negatives and inconsistent results. While modifications to the CTAB (Cetyltrimethylammonium bromide) protocol have shown promise in improving DNA quality (Schenk et al., 2023, Varma et al., 2007).

Previous studies have employed various DNA-based methods for authenticating *Terminalia* and *Phyllanthus* species using leaf tissue. *ITS2* has been used for Indian *Terminalia* (Nathaniel & Parani, 2016), while PCR-RFLP and ARMS-PCR were applied to Thai *Terminalia* crude drugs (Intharuksa et al., 2016). Authentication of *Phyllanthus* has relied on RAPD-SCAR or ITS-SCAR approaches (Dnyaneshwar et al., 2006). These methods demonstrate the utility of DNA-based techniques for accurate species identification.

Isolating high-quality, amplifiable DNA from TB, TC and PE fruit tissue or powder is challenging due to high polyphenol content. This study presents an improved DNA-based authentication method for TB, TC, and PE dried fruit powder. We developed an optimized protocol with tailored buffering strength and polyvinylpyrrolidone (PVP) concentration to address DNA extraction challenges. The method's effectiveness was rigorously validated using species-specific PCR, digital PCR, HPTLC analysis of gallic acid and commercially available Baheda (TB), Harde (TC) and Amla (PE) fruit powders. This comprehensive approach ensures the accuracy and reliability of the DNA-based method, advancing quality control and authentication of herbal products.

2.2 Material and Methods

2.2.1 Plant Material Collection, DNA Isolation, and *rbcL* Gene Sequencing

TB, TC and PE leaf and fruit samples were collected from the Directorate of Medicinal and Aromatic Plants Research, Anand, Gujarat, India, with taxonomist assistance. Herbarium vouchers (TB = BG-201130-0011; TC = BG-201130-0012; PE = BG-201130-0013) were prepared and deposited in the institutional herbarium. Leaf DNA was extracted 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 2.1). Correct species assignment was obtained in the BLAST results and sequences submitted to the NCBI database with accession numbers MW628925, MW628910, and MW628927 for TB, TC, and PE, respectively. Leaf samples were collected to compare PCR assay sensitivity with polyphenol-rich dried fruit tissue from the same plants. 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.

2.2.2 Optimization and Selection of DNA Extraction Protocol from Dried Fruits and Market Products

Eleven different DNA isolation protocols were evaluated to optimize DNA extraction from dried fruit samples of TB, TC and PE (Table 2.2). The initial approach utilized a commercially available DNeasy Plant Mini Kit (Qiagen, India) as the baseline method,

following the manufacturer's instructions. To address polyphenol challenges, Protocols 2 and 3 used a modified CTAB buffer with increased PVP (100 mg) (Warude et al., 2003), differing in purification (isopropanol precipitation vs. column-based). Protocols 4 and 5 adjusted lysis buffer pH with increased Tris-Cl (0.2 M and 0.5 M, respectively). Protocol 6 added a phenol treatment step before phenol: chloroform: isoamyl alcohol (PCI) alcohol extraction. Protocols 7 and 8 incorporated pre-lysis soaking (1 hour and overnight). Protocols 9-11 tested varying PVP amounts (200 mg, 500 mg, 1000 mg) during grinding. Protocol 11, using 1 g PVP, yielded the highest DNA quality and was selected for subsequent extractions (Table 2.2).

This optimized protocol involves the following steps:

1. **Sample Preparation and Lysis:** One hundred milligrams of dried fruit powder were combined with 1 g of polyvinylpyrrolidone and finely ground using liquid nitrogen. The ground mixture was then transferred to a tube containing 3 mL of optimized extraction buffer [0.5 M Tris-Cl (pH 8.0), 20 mM EDTA (Ethylenediaminetetraacetic acid) disodium salt (pH 8.0), 1.5 M NaCl, 4.0% Cetyltrimethylammonium Bromide (CTAB) (w/v), 5% β-mercaptoethanol (v/v), and 2% Polyvinylpyrrolidone (PVP) (w/v)]. The mixture was incubated at 65 °C for 30 minutes in a water bath with shaking at 10-minute intervals to facilitate cell lysis.
2. **Phenol Extraction:** After incubation, 1 mL of phenol was added to the supernatant lysate, mixed thoroughly, and centrifuged at 15,000 x g for 10 minutes at 4°C. The aqueous phase, containing the DNA, was carefully transferred to a fresh tube.
3. **Phenol: Chloroform: Isoamyl (PCI) Alcohol Treatment:** To further purify the DNA, 1 mL of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the aqueous phase, mixed well, and centrifuged at 15,000 x g for 5 minutes at 4°C. This step was repeated to ensure thorough removal of contaminants.
4. **Silica Column Purification:** The DNA was then purified from the aqueous phase using a silica column, following the DNeasy Plant Mini Kit (Qiagen, India), protocol from the binding step onwards. This step removes any remaining impurities, resulting in high-quality DNA.

5. **DNA Quantification:** Finally, the concentration and purity of the extracted DNA were measured using the Qubit 4.0 fluorometer with the 1X HS DNA assay kit (Thermo Fisher Scientific, USA).

2.2.3 DNA Quality Evaluation using *ITS2* Metabarcode

The quality of extracted DNA from TB, TC and PE fruits, and their market samples, was assessed by PCR amplification of the *ITS2* region. The *ITS2* region was chosen for amplification and evaluation of potentially sheared DNA extracted from fruit tissues. Its shorter length facilitates amplification even when DNA is fragmented. Furthermore, species-specific primers targeting the *ITS*-SCAR region were utilized for TB, TC and PE. The *ITS2* metabarcode primers and thermal cycler conditions were described in Chapter 3, section 3.2.3.

2.2.4 Species-Specific PCR Assay

For the species-specific assays, primers for TB and TC were sourced from Sharma and Shrivastava, (2016), while primers for PE were from Bandyopadhyay et al., (2010). The 20 µL PCR reaction mixture consisted of 10 µL Emerald Takara master mix (2X), 2 µL DNA (5 ng/µL), 1 µL each of forward and reverse primers (5 pmol), 2 µL BSA (2 mg/mL), and 4 µL PCR-grade water (Table 2.3a). Thermal cycling conditions were: 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 30 seconds, 52 °C/ 56 °C/ 60 °C for 30 seconds, and 72 °C for 30 seconds, with a final extension at 72°C for 5 minutes (Table 2.3b). A sensitivity assay was performed using a DNA concentration gradient (10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.07, and 0.01 ng) with species-specific primers for TB, TC, and PE, using both leaf and fruit DNA. Species-specific PCR assays were performed to authenticate market formulations. The same PCR conditions were used for all species-specific primer sets in the sensitivity assay.

2.2.5 Digital PCR (dPCR)

The dPCR (Digital PCR) reaction mixture was constituted with 13.7 µL of Evagreen master mix (3X) from (QIAcuity EG PCR Kit, Qiagen, India), 1.0 µL of forward and reverse primers (10 pmol each), 2.0 µL of DNA template, 1.0 µL of *Eco*R1 (1U/µL) and 20.1 µL of nuclease-free water was added in a total of 40 µL volume (Table 2.4a). The reaction was incubated for 10 minutes at room temperature for DNA digestion and then loaded to a QIAcuity Nanoplate 26k 24-well plate (Qiagen, India). The plate was

run in the QIAcuity Digital PCR System, (Qiagen, India). The thermal cycling conditions were as follows; initial denaturation at 95 °C for 2 minutes, then 40 cycles of 95 °C for 15 seconds, 56 °C for 30 seconds, and 72 °C for 40 seconds, and a final cooling down at 40 °C for 5 minutes (Table 2.4b). 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) x obtained copy numbers/µL] as per the guideline given in the QIAcuity® user manual extension. A sensitivity assay was performed using a DNA concentration gradient (10.0, 5.0, 2.5, 1.25, 0.6, 0.3, 0.15, and 0.07 ng) with species-specific primers for TB, TC and PE, using their respective fruit DNA.

2.2.6 Validation of Market Formulation with HPTLC of Gallic Acid

Gallic acid was used for the phytochemical-based identification of the components using High-performance thin-layer chromatography (HPTLC) (API, Part I, volume IV). Standard was prepared by mixing 1 mg of gallic acid (Sigma-Aldrich, India) in 1 ml of methanol. For the samples, one gram of the powder of each fruit was extracted in 10 ml of methanol, and 10 µl of each was spotted on a TLC plate (MERCK Millipore Silica gel 60 F254; Camag HPTLC System) and run in the mobile phase; Toluene: Ethyl acetate: Formic acid (2: 5: 1.5). TLC plates have been visualized at 254 nM using Camag TLC Scanner.

2.3 Results and Discussion

2.3.1 Improvisation of PCR Amplifiable DNA isolation from TB, TC, and PE Fruit Powder using Additives

TB, TC and PE fruits are of potent multi-therapeutic ayurvedic formulations with high cultivation and consumption economics (Goraya & Ved, 2017). Various pharmacopeia has 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 & Shaw, 2022). The low quality and quantity of PCR-amplifiable DNA pose major challenges in authenticating raw materials. Additionally, there is no universal DNA isolation protocol that works for all plant species and tissue types. Researchers continue to face difficulties in obtaining PCR-amplifiable DNA.

Therefore, the objective of the present study was to develop a protocol to extract high-quality, homogeneous PCR-amplifiable DNA from polyphenolic-rich fruits, strengthening 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 a 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 yields were obtained for all three fruits. Notably, the *ITS2* metabarcode gave positive amplification only for the TB fruit (Table 2.5; Figure 2.1a).

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 PVP during tissue maceration has been documented to effectively scavenge polyphenols during tissue lysis (Porebski et al., 1997). Additionally, a higher concentration of 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 CI 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 2.2. During the lysis phase, an increase in pH was observed, potentially attributed to oxidative reactions. This pH fluctuation was intermittently stabilized throughout the incubation period, as reported by 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 2.5; Figure 2.3b, 2.3c).

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 ug/g for TB, TC and PE fruit tissue (Table 2.5). *ITS2* metabarcode amplification results revealed that PCR amplifiability was

observed in PE and TB fruit DNA (Figure 2.1a, 2.1c), but no amplification was observed in the case of TC fruit DNA (Figure 2.1b).

The lysates, particularly those from PE and TC, exhibited notable viscosity after incubation. This viscosity could likely be attributed to the formation of complexes between PVP-polyphenol and CTAB-polysaccharide, which were not eliminated during the PCI and CI 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 is 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 2.5; Figure 2.1b).

The PE dry fruit tissue was the most challenging to grind compared to the TB and TC fruits. This indicated the presence of higher levels of polysaccharides and polyphenolic compounds in the PE fruit, which could potentially inhibit the enzymatic activities required for DNA isolation and subsequent molecular analysis (Aboul-Maaty & Oraby, 2019). To address the challenging tissue texture, a soaking-out technique was employed to soften the tissue through two protocols (7 and 8) before DNA extraction from the dried TB, TC, and PE fruits (Asish et al., 2010; Singh et al., 1999). Protocol 7 involved a 1-hour incubation, while protocol 8 involved an overnight incubation at room temperature. The results revealed increased PE fruit DNA concentrations of 2.68 and 4.4 μg/g for protocols 7 and 8, respectively (Table 2.5). 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 (Figure 2.1b).

The pronounced browning observed in TC fruit DNA during the elution step in protocols 1-8 suggests a co-precipitation of polyphenols with the DNA. This interaction has the potential to hinder the efficiency of PCR amplification, potentially impeding 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 2.5; Figure 2.1a, 2.1b, 2.1c). A charcoal-based DNA isolation method from mature leaves, embryos and bark of TB, the bark of TC was developed by (Sharma et al., 2017). In these studies, DNA extracted from optimized protocols showed PCR amenabilities for various polymorphic markers. However, we were not able to amplify DNA through PCR with *ITS* primers (Table 2.1) from fruits of TB, TC, PE might be due to highly acidic pH, high polyphenolic and polysaccharide content and secondary metabolites content of fruits compared 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 the high polyphenols present in the species and its fruit tissue. The polyphenol readily reacts with DNA and is responsible for the browning effect and degradation of DNA due to acidic pH. The use of high amounts of PVP during the grinding scavenges the polyphenols and the high molarity of the Tric-Cl buffering strength stabilizes the pH and can maintain the pH. While for PE the real challenge was high tannin and polysaccharide content present in fruit tissue.

2.3.2 Species-specific PCR Assay Optimization

The species-specific PCR assay conditions were optimized using the respective leaves DNA of TB, TC and PE. The results revealed that 56 ºC is the optimal temperature for the primer annealing (Figure 2.2). DNA isolated from fruits of TB, TC and PE using eleven protocols was subjected to optimized species-specific PCR assay. The DNA extracted from TB fruits using all 11 protocols was successfully amplified using a species-specific assay (Figure 2.3a). While in the case of the TC, amplification was observed only with three protocols i.e. protocol number ID of 9 to 11 (Figure 2.3b). Protocols number 5 to 11 gave positive amplification for the PE fruit DNA (Figure 2.3c). 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 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, for protocols 8 and 9, respectively (Figure 2.3b). The results demonstrated that a high amount of PVP during grinding reduced browning of the DNA eluate and improved PCR amplification of TC fruit DNA.

A sensitivity assay determined 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. (Figure 2.4a, 2.4b, 2.4c, 2.4d, 2.4e, 2.4f). 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 the band on gel for all plants. Similarly, in the case of the fruits, the results revealed that 0.01 ng DNA input gives amplification for TB (Figure 2.4d). While, in the case of TC and PE fruit DNA, 0.15 ng of minimal DNA concentration was required to observe amplification on agarose gel (Figure 2.4e, 2.4f). 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. (Figure 2.4b, 2.4c, 2.4e, 2.4f). This 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 TB, TC and PE labeled as Baheda (TB fruit), Harde (TC fruit), and Amla (PE fruit) from the local market. PCR amplification was performed using both *ITS2* metabarcode and species-specific primers as described in section 2.2.4. Successful PCR amplification was observed with all the TB fruit (Baheda) powder products (Figure 2.5a, 2.6a). In the case of TC (Harde) powder products, positive amplification was observed in five samples (Figure 2.6b), whereas

only three samples exhibited positive amplification for PE (Amla) powder products (Figure 2.6c). Nevertheless, all fruit powders exhibited positive amplification with the *ITS2* metabarcode (Figure 2.5b, 2.5c). 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, Sharma, et al., 2022). Only 22.2 % of Bramhi (*Bacopa monnieri* / *Centella asiatica*) powder 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 adulteration with a 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.

2.3.3 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 TB, TC and PE DNA fruits. In dPCR, the DNA is distributed across the partitions, along with secondary metabolites, thereby enhancing the precision, accuracy, and robustness of the analysis. 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 ng 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, 1,230 to 25 for TC fruit DNA, and 1,905 to 16 for PE fruit DNA were obtained against 10 to

0.07 ng DNA input (Table 2.6). The results indicated the positive correlation of increasing DNA input with positive valid partitions (Figure 2.7a, 2.7b, 2.7c). The linear regression correlation coefficient between DNA concentration and copy numbers was obtained to assess the dynamic range, for the DNA extracted from the fruits of TB, TC and PE. The obtained results depicted $R^2 > 0.99$ for all three fruits, henceforth we found a 10 ng to 0.07 ng optimal dynamic range for all three fruits of TB, TC and PE, as illustrated in (Figure 2.8, 2.8b, 2.8c). 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 twofold 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 gave up to 0.001 ng sensitivity for DNA obtained *O. sanctum* and *O. basilicum* (Travadi, Sharma, et al., 2022), while for 0.06 ng DNA sensitivity was observed for DNA of P. *nigrum* and *C. papaya* (Travadi, Shah, et al., 2022). Similar studies have been also reported in other plants by 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. Overall, our results indicate that the improved DNA isolation protocol for the dried fruit powder significantly improved the quality of DNA, which is reflected in 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.

2.3.4 HPTLC with Gallic acid

Gallic acid, a reference chemical marker, in TB, TC, and PE, mentioned in API, Vol IV, was subject to HPTLC analysis to validate DNA-based authentication using the standard analytical method. Each extracted sample from the TB, TC, PE fruit and powders underwent HPTLC analysis. HPTLC analysis, using gallic acid as a standard, confirmed its presence in TB, TC, and PE powders, evidenced by the consistent Rf value of 0.3 observed across all samples and the standard (Figure 2.9a, 2.9b, 2.9c). In the species-specific PCR analysis, some market samples of Harde (H4) and Amla (A1, A3, A4) failed to amplify. However, HPTLC analysis detected gallic acid in these same samples. This discrepancy may be due to limitations of the DNA-based method, such

as insufficient DNA quality or quantity, or the high sensitivity and specificity of the DNA-based method compared to HPTLC with gallic acid. The use of a common reference marker (gallic acid) for all three plants in the HPTLC analysis could lead to false-positive detections. A combined approach using complementary analytical methods like HPTLC strengthens authentication by providing orthogonal validation.

2.4 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 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. The successful detection of gallic acid via HPTLC supports the DNA-based findings, offering a complementary method for authentication. Further, digital PCR is found to excel in sensitivity, robustness, and accuracy, while conventional PCR can be considered as a more cost-effective method.

Table 2.1 Details about Species-specific and universal primers.

Plant Species	Primer	Sequence (5' to 3')	Annealing Temperature	Amplicon Length	References
T.bellirica	TB-F	GTCGATCTAAG CCCCAGCAG	56º C	150 bp	Sharma & Shrivastava 2016
T.chebula	TC-F	GATGGGAGGAT GGTCCGGGA		244 bp	
ITS4	ITS4-R	TCCTCCGCTTAT TGATATGC			
P.emblica	PE-F	TTTAGTCACTGC GGATGGTG	56º C	212 bp	Bandyopadhyay &Raychaudhuri, 2010
	PE-R	GAGATATCCGTT GCCGAGAG			
rbcLa	*rbcL*a-F	ATGTCACCACA AACAGAGACTA AAGC	60º C	600 bp	Maloukh et al., 2017
	*rbcL*a-R	GTAAAATCAAG TCCACCRCG			
ITS2 metabarcode	*ITS2*-F	CRRAATCCCGTG AACCATCGAGT CYT	60º C	310-330 bp	Travadi et al., 2023
	ITS2-R	AGCGGGTRRTC CCRCCTGACYTG			

Table 2.2 Optimization of DNA Isolation Protocols form dried fruit tissue.

DNA isolation											
		Lysis Procedure							**DNA purification**		
		CTAB buffer composition									
Protocol number	**Tissue soaking procedure (hr.)**	CTAB (%)	Tris-Cl (M)	EDTA (mM)	NaCl (M)	PVP (%)	BME (%)	**PVP during the grinding (mg)**	**Phenol treatment**	**PCI$ treatment**	**C / I**
1	No	DNeasy plant mini kit (QIAGEN, India)							No	No	No
2	No	4	0.1	20	1.4	2	5	100	No	Yes	I
3	No	4	0.1	20	1.4	2	5	100	No	Yes	C
4	No	4	0.2	20	1.4	2	5	100	No	Yes	C
5	No	4	0.5	20	1.4	2	5	100	Yes	Yes	C
6	No	4	0.5	20	1.4	2	5	100	Yes	Yes	C
7	1	4	0.5	20	1.4	2	5	100	Yes	Yes	C
8	24	4	0.5	20	1.4	2	5	100	Yes	Yes	C
9	No	4	0.5	20	1.4	2	5	200	Yes	Yes	C
10	No	4	0.5	20	1.4	2	5	500	Yes	Yes	C
11	No	4	0.5	20	1.4	2	5	1000	Yes	Yes	C

PCI [$] - Phenol Chloroform Isoamyl Alcohol, Column purification (C), Isopropanol precipitation (I)

Table 2.3a Conventional PCR reaction set up using species specific primer.

Component	**Volume (µL)**
Evagreen Master Mix (2X)	10.0
Template DNA	2.0
*Forward Primer (5 pmol)	1.0
*Reverse Primer (5 pmol)	1.0
BSA (2 mg/mL)	2.0
PCR-grade Water	4.0

*Forward and reverse species-specific primer pairs are used for TB, TC, and PE as mentioned in Table 3.1.

Table 2.3b Conventional PCR cycling condition for species specific primer.

Steps	**Temperature (°C)**	**Duration**	**Number of Cycles**
Initial Denaturation	95	5 minutes	1
Denaturation	95	30 seconds	35
Annealing	56		
Extension	72		
Final Extension	72	5 minutes	1

Table 2.4a Digital PCR reaction set up using species specific primer.

Component	**Volume (µL)**
Emerald Takara Master Mix (3X)	13.3
Template DNA	2.0
*Forward Primer (10 pmol)	1.0
*Reverse Primer (10 pmol)	1.0
EcoR1 (1U/µL)	1.0
PCR-grade Water	21.7

*Forward and reverse species-specific primer pairs are used for TB, TC, and PE as mentioned in Table 3.1.

Table 2.4b Digital PCR cycling condition for species specific primer.

Steps	**Temperature (°C)**	**Duration**	**Number of Cycles**
Initial Denaturation	95	5 minutes	1
Denaturation	95	15 seconds	40
Annealing	56	30 seconds	
Extension	72	40 seconds	
Final Extension	40	5 minutes	1

Table 2.5. DNA quantification and PCR assay results from optimization protocols listed in Table 2.2.

Protocol ID	TB			TC			PE		
	D(µg/g)	IP	SP	D(µg/g)	IP	SP	D(µg/g)	IP	SP
1	TL	+	+	TL	-	-	TL	-	-
2	0.32	+	+	0.04	-	-	0.28	-	-
3	0.68	+	+	0.12	-	-	0.32	-	-
4	3.64	+	+	0.20	-	-	0.44	-	-
5	9.84	+	+	0.79	-	-	1.04	+	+
6	10.00	+	+	1.28	-	-	1.52	+	+
7	9.36	+	+	0.86	-	-	2.68	+	+
8	10.84	+	+	0.79	-	-	4.40	+	+
9	11.56	+	+	3.74	+	+	5.68	+	+
10	13.84	+	+	5.60	+	+	7.44	+	+
11	18.00	+	+	12.00	+	+	9.32	+	+

D- DNA concentration (µg/g), IP- *ITS2* PCR amplification, SP- Species-specific PCR amplification, TB- Fruit of *T. bellirica*, TC- Fruit of *T. chebula*, PE- fruit of *P. emblica*, TL- Too Low

Table 2.6 Results of digital PCR.

Fruit DNA	DNA input (ng)	Target gene copies/µL	Total copy numbers	Average copy numbers	Total valid partitions	Positive partitions	Negative
***Terminalia bellirica* (TB)**	10	8144.4	166965	171674	25044	24983	61
		8819.1	176382		25063	25018	45
	5	5146.7	102934	101577	25394	24814	580
		5011	100220		25453	24725	728
	2.5	1930.4	38608	41576	25443	19300	6143
		2227.2	44544		25462	20257	5205
	1.2	1218.8	24376	24775	25447	15085	10362
		1258.7	25174		25507	15155	10352
	0.6	634.3	12686	12828	25164	9447	15717
		648.5	12970		25221	9471	15750
	0.3	323.8	6476	6294	25371	5454	19917
		305.6	6112		25334	5101	20233
	0.15	190	3800	3718	25348	3342	22006
		181.8	3636		25453	3171	22282
	0.07	111.8	2236	2328	25458	1992	23466
		121	2420		25481	2145	23336
***Terminalia chebula* (TC)**	10	66.37	1327	1230	25150	1215	23935
		56.58	1132		25185	1068	24117
	5	27.44	549	539	25417	507	24910
		26.41	528		25465	492	24973
	2.5	13.9	278	253	25497	260	25237
		11.39	228		25502	213	25289
	1.2	7.374	147	124	25471	140	25331
		5.039	101		25454	95	25359
	0.6	3.518	70	67	25481	66	25415
		3.195	63		25495	58	25437
	0.3	3.082	61	60	25476	57	25419
		2.88	57		25498	54	25444
	0.15	2.354	47	43	25519	43	25476
		1.914	38		25492	35	25457
	0.07	1.43	28	25	25465	27	25438
		1.027	20		25479	19	25460
***Phyllanthus emblica* (PE)**	10	95.25	2193	1905	25485	2427	23058
		80.84	1617		25448	1432	24016
	5	43.54	871	924	25452	792	24660
		48.86	977		25436	898	24538
	2.5	26.04	521	488	25435	488	24947
		22.75	455		25435	420	25015
	1.2	11.92	238	226	25443	220	25223
		10.69	214		25459	197	25262
	0.6	7.13	143	143	25458	130	25328
		7.15	143		25464	132	25332
	0.3	3.987	79.74	70	25494	72	25422
		2.972	59.44		25498	55	25443
	0.15	1.758	35.16	31	25481	33	25448
		1.35	27		25484	25	25459
	0.07	0.746	14.92	16	25514	14	25500
		0.825	16.5		25516	15	25501

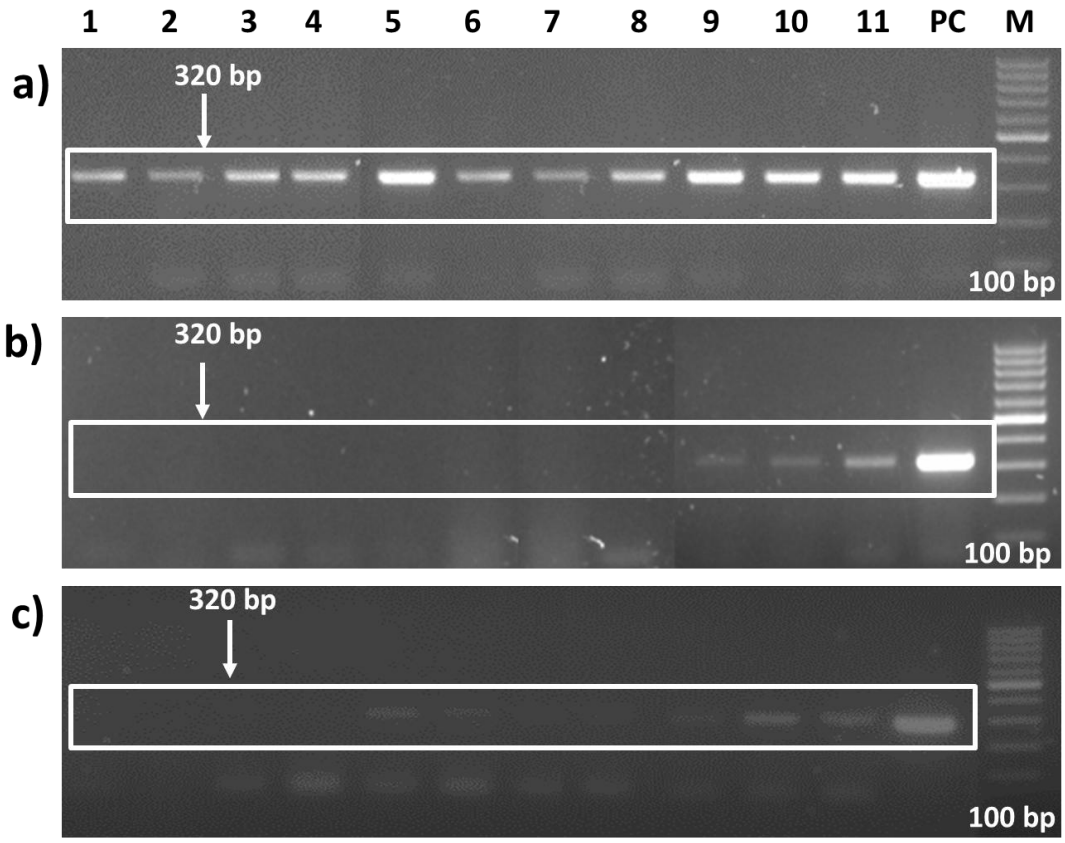

Figure 2.1 *ITS2* metabarcode amplification using 11 different DNA optimization protocols. a), b), and c) are *Terminalia bellirica* (TB), *Terminalia chebula* (TC) and *Phyllanthus emblica* (PE). 1-11 = protocol ID; PC (Positive control) = DNA isolated from respective leaf tissue; M = 100 bp DNA marker (100 -1000 bp). Expected amplicon sizes: *ITS2*: 320 bp.

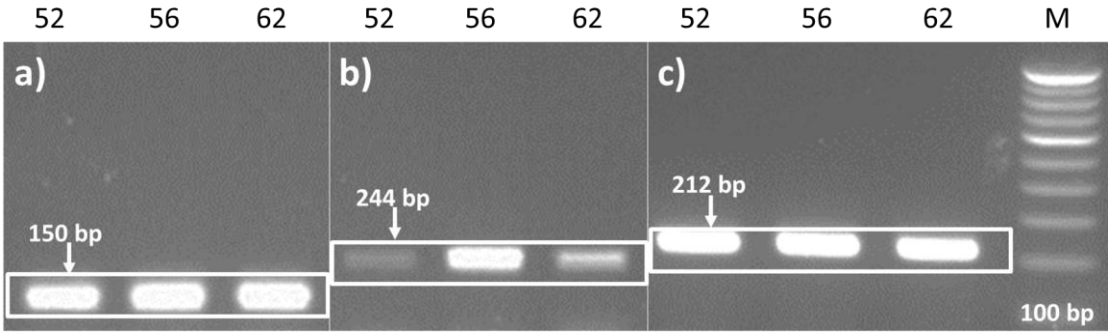

Figure 2.2 Optimization of Species-specific primers using leaf DNA at 52, 56, and 62 °C annealing temperature. a), b), and c) are *Terminalia bellirica* (TB), *Terminalia chebula* (TC) and *Phyllanthus emblica* (PE). M= 100 bp DNA marker (100 -1000 bp). Expected amplicon sizes: TB: 150 bp, TC: 244 bp, PE: 212 bp.

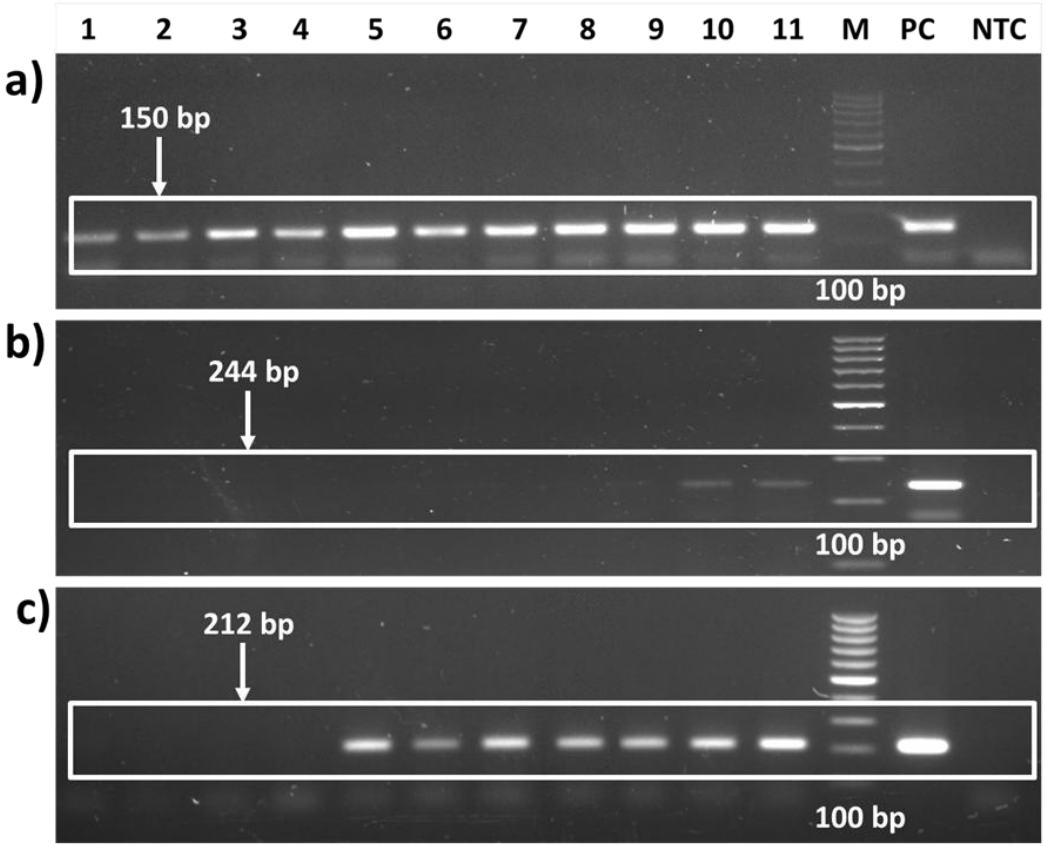

Figure 2.3 Species-specific PCR amplification using 11 different DNA optimization protocols. a), b), and c) are *Terminalia bellirica* (TB), *Terminalia chebula* (TC) and *Phyllanthus emblica* (PE). NTC = No Template Control; M= 100 bp DNA marker (100 -1000 bp); PC (Positive control): DNA isolated from respective leaf tissue. Expected amplicon sizes: TB: 150 bp, TC: 244 bp, PE: 212 bp.

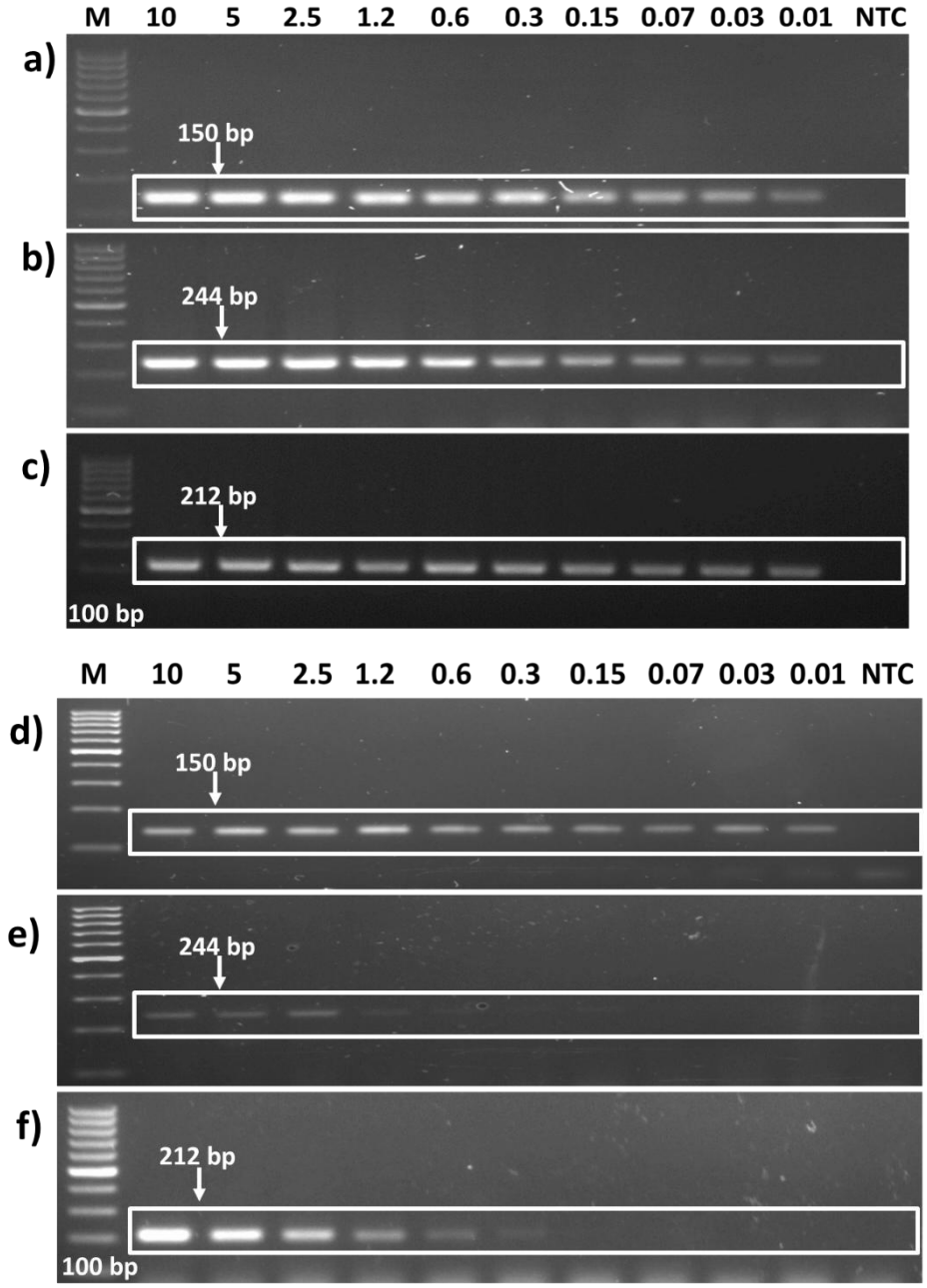

Figure 2.4 Sensitivity assay using 10 ng to 0.01 ng DNA input from leaf (a-c) and dried fruit (d-f) tissues of *Terminalia bellirica* (TB), *Terminalia chebula* (TC), and *Phyllanthus emblica* (PE) with species-specific primers. NTC = No Template Control; M= 100 bp DNA marker (100 -1000 bp). Expected amplicon sizes: TB: 150 bp, TC: 244 bp, PE: 212 bp.

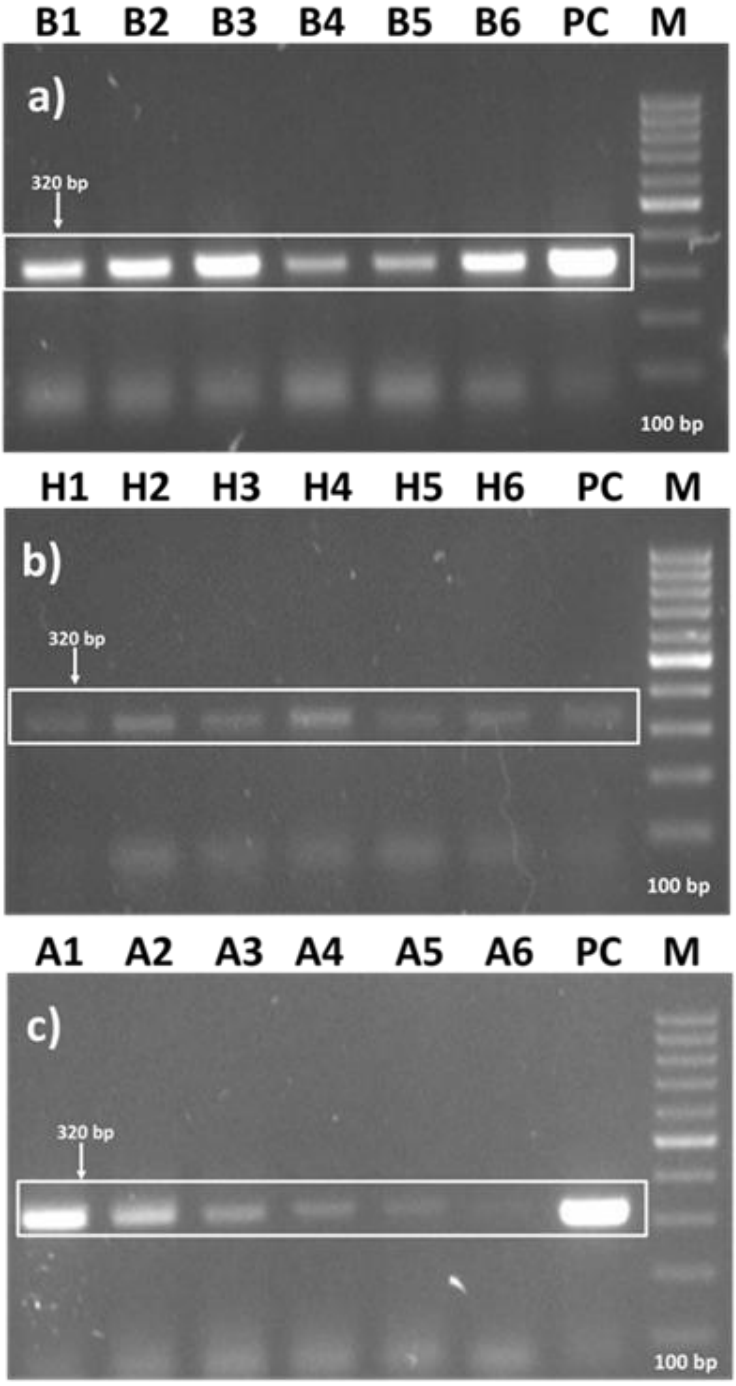

Figure 2.5 Evaluation of DNA quality using *ITS2* metabarcode for the authentication of single-drug market formulations. a) *Terminalia bellirica* fruit powder (B1-B6), b) *Terminalia chebula* fruit powder (H1-H6), and c) *Phyllanthus emblica* fruit powder (A1-A6). PC (Positive control): DNA isolated from respective dried fruit tissue; NTC: No Template Control; M: 100 bp DNA marker (100-1000 bp). Expected amplicon sizes: *ITS2*: 320 bp.

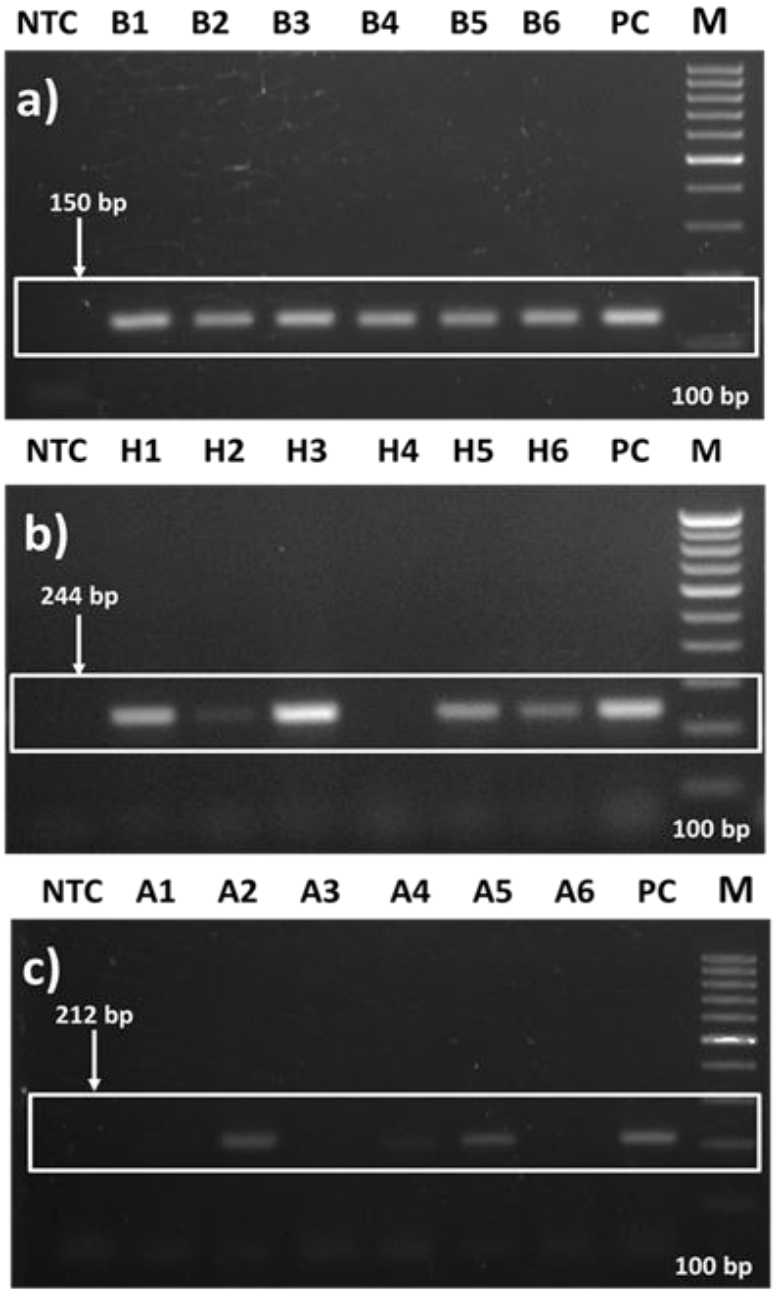

Figure 2.6 Evaluation of a species-specific PCR assay for the authentication of single-drug market formulations. a) *Terminalia bellirica* fruit powder (B1-B6), b) *Terminalia chebula* fruit powder (H1-H6), and c) *Phyllanthus emblica* fruit powder (A1-A6). PC (Positive control): DNA isolated from respective dried fruit tissue; NTC: No Template Control; M: 100 bp DNA marker (100-1000 bp). Expected amplicon sizes: TB: 150 bp, TC: 244 bp, PE: 212 bp.

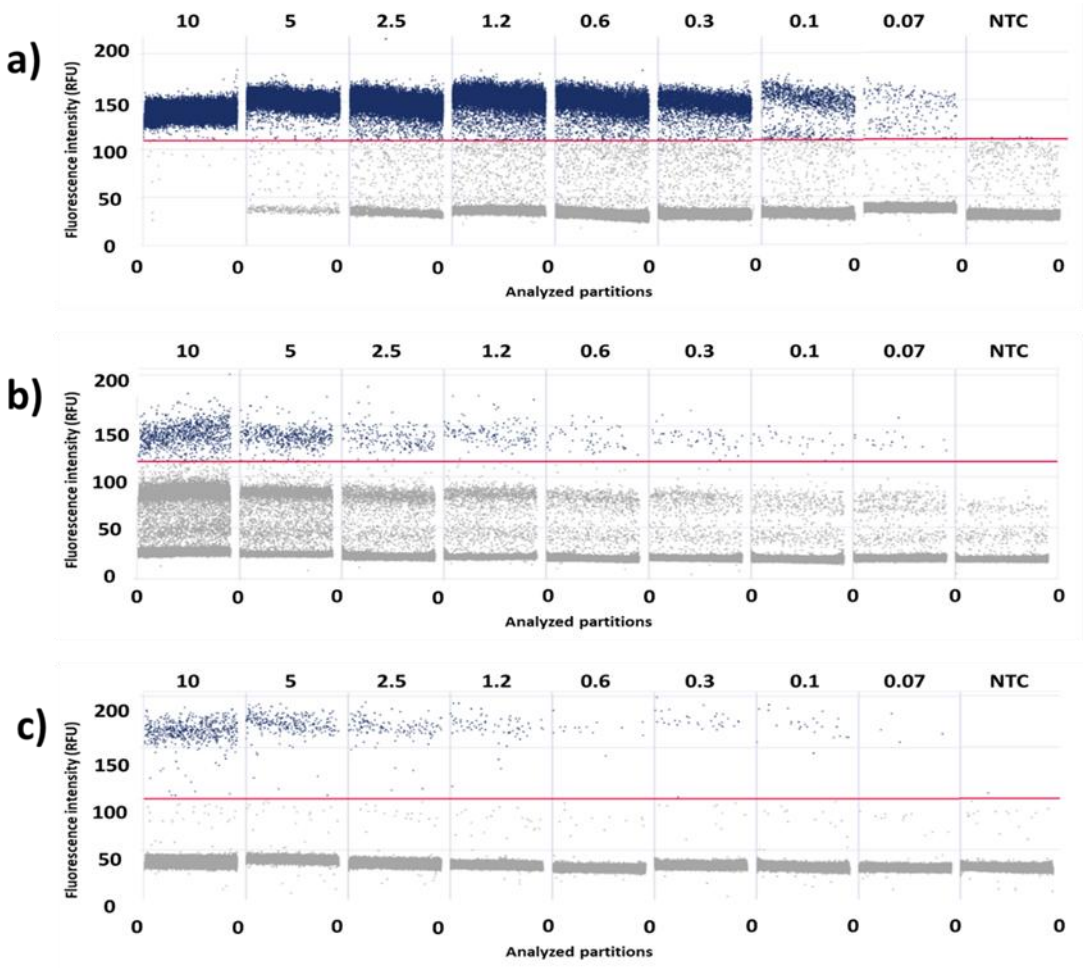

Figure 2.7 Digital PCR assay using a) *Terminalia bellirica* (TB), b) *Terminalia chebula* (TC), and c) *Phyllanthus emblica* (PE) fruit DNA. The red line represents the fluorescence amplitude threshold. Positive partitions were observed above the threshold and negative partitions were below the threshold. NTC = No Template Control.

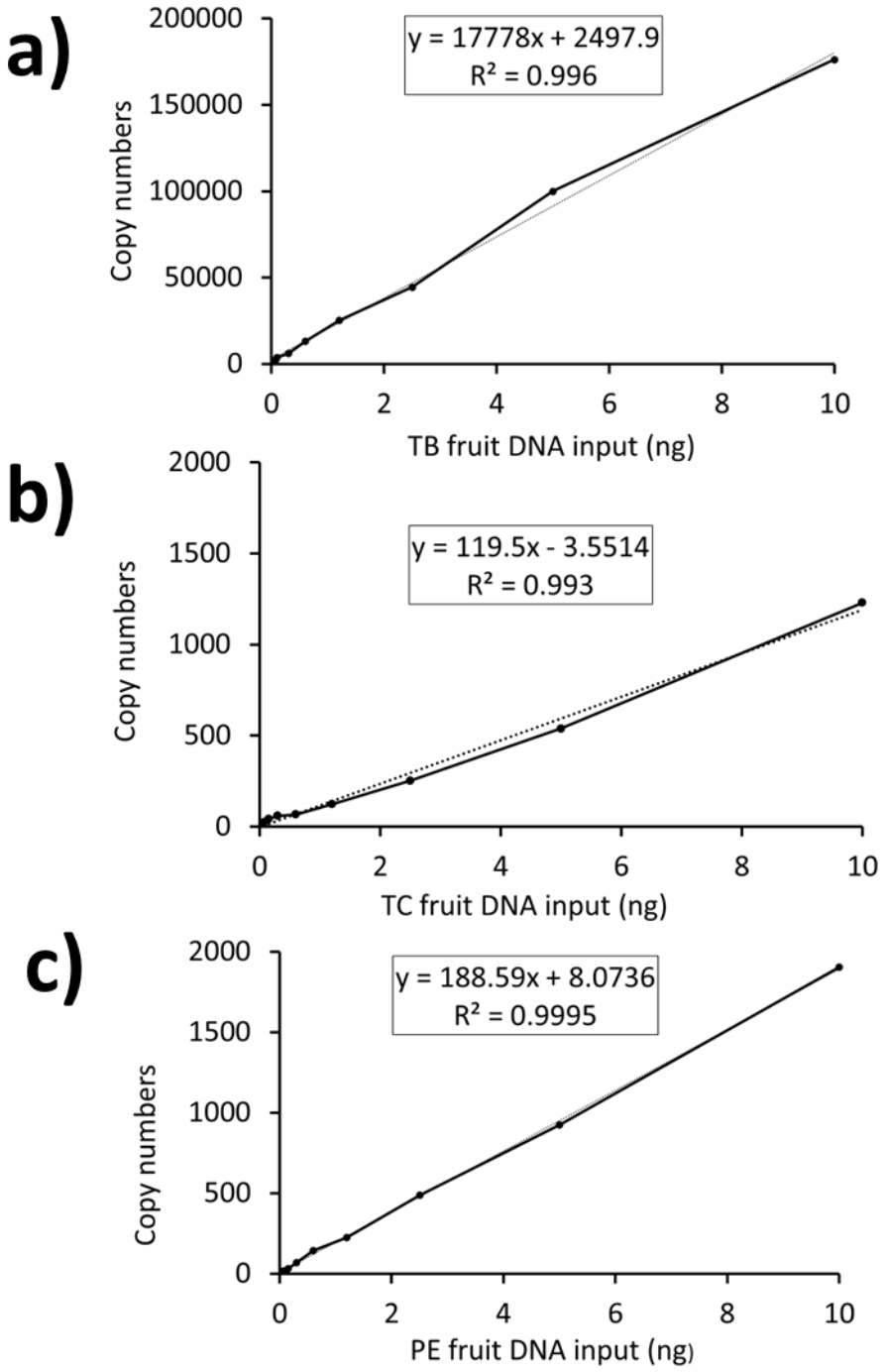

Figure 2.8 Linear Relationship Between DNA Concentration (ng) and Corresponding DNA Copy Numbers in a) *Terminalia bellirica* (TB), b) *Terminalia chebula* (TC) and c) *Phyllanthus emblica* (PE) fruit DNA using dPCR. Regression equations indicate 'y' (copy numbers), 'x' (DNA input), and 'R²' (coefficient of determination).

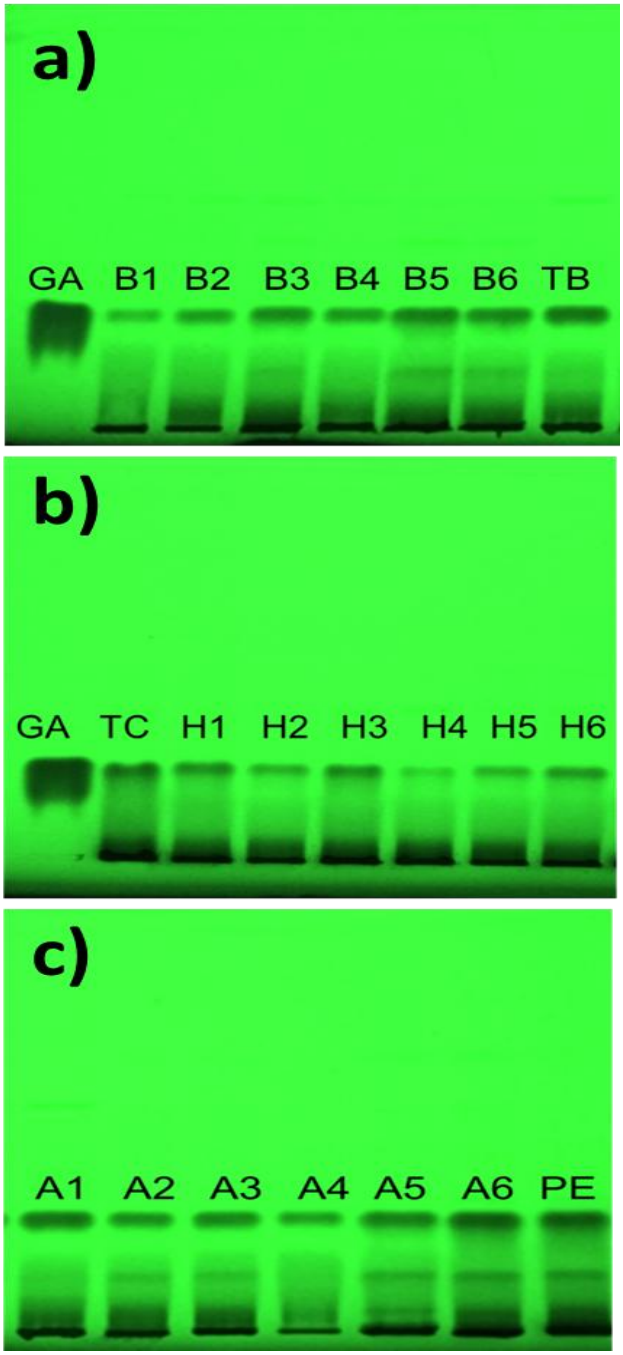

Figure 2.9: HPTLC analysis of gallic acid in single-drug market formulations: a) *Terminalia bellirica* (TB), B1–B6 (Baheda Powder); (b) *Terminalia chebula* (TC), H1–H6 (Harde Powder); (c) *Phyllanthus emblica* (PE), A1–A6 (Amla Powder). TB, TC, and PE = Positive controls; GA = Gallic acid reference standard.