Publications and Presentations

International Journals

1. **Travadi, T**., Sharma, S., Pandit, R., Joshi, C., Joshi, P., & Joshi, M. (2024). Overcoming Challenges in DNA Extractions from Triphala Ingredients: A Way Forward for Optimization of Conventional and Digital PCR Assays for Molecular Authentication. *Food Analytical Methods*, 1-11.
2. **Travadi, T.,** Sharma, S., Pandit, R., Joshi, C., Joshi, M., & Joshi, P. (2024). Advancing Herbal Product Authentication: A Comprehensive Review Of DNA-Based Approach for Quality Control and Safety Assurance. *Educational Administration: Theory and Practice*, *30*(6 (S)), 15-25.

International Conferences

1. Oral Presentation at the 2nd Multi-Disciplinary International Conference, GTU ICON 2022, Under the title of "Authentication of *Phyllanthus Emblica* (Amla), *Terminalia Chebula* (Harde), and *Terminalia Bellerica* (Baheda) using species-specific PCR Assay" on 23rd September 2022.
2. Oral presentation at 2nd International Conference on Emerging Trends & Contemporary Practices (ICETCP-2024), Under the title of "Advancing Herbal Product Authentication: A Comprehensive Review of DNA-Based Approach for Quality Control and Safety Assurance" on 09 – 10th February 2024.
3. Poster presentation at IISF-2023, Bhopal, Under the title of "Validation of newly designed *rbcL* and *ITS2* metabarcodes for detecting medicinal plants species using DNA metabarcoding in predefined mock controls" on 21 to 24th January, 2023.

National Conferences

1. Poster presentation at Bhartiya Vigyan Sammelan (BVS), under the title of "Optimization of DNA Isolation from Polyphenol-Rich Dried Fruits: Enabling Herbal Authentication Through Conventional and Digital PCR'', 21st to 24th December, 2023.
2. Oral presentation at the 29th IAAT Annual Conference and National Symposium on Modern Trends in Biosystematics of Angiosperms, TBGRI, Trivandrum under the title of "Identification of botanical adulterants in Triphala using species-specific primers and Metabarcoding" on 11-13th November 11-13, 2019.

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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	TB			TC			PE		
		CTAB buffer composition						PVP during the grinding (mg)	Phenol treatment	PCI[$] treatment	Column purification (c)/ isopropanol precipitation (I)	D	IP	SP	D	IP	SP	D	IP	SP
		CTAB (%)	Tric-Cl (M)	EDTA (mM)	NaCl (M)	PVP (%)	BME (%)													
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

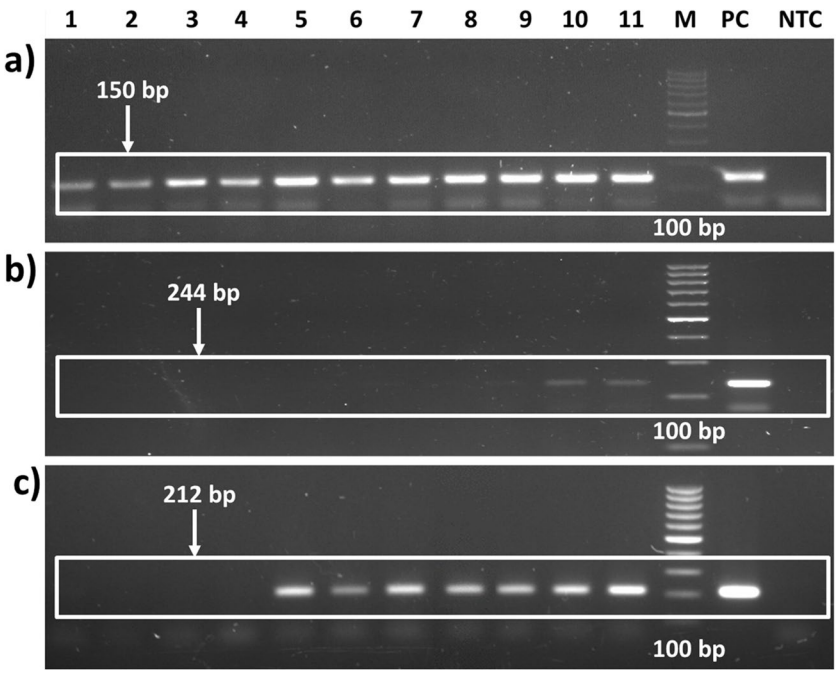

Fig. 1 Species-specific PCR assay with 11 different protocols of DNA isolation from fruits. **a** *Terminalia bellirica* (TB), **b** *Terminalia chebula* (*TC*), and **c** *Phyllanthus emblica* (*PE*); 1–11 = Protocol ID; PC = DNA isolated from respective leaves; NTC = no template control; M = 100 bp 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

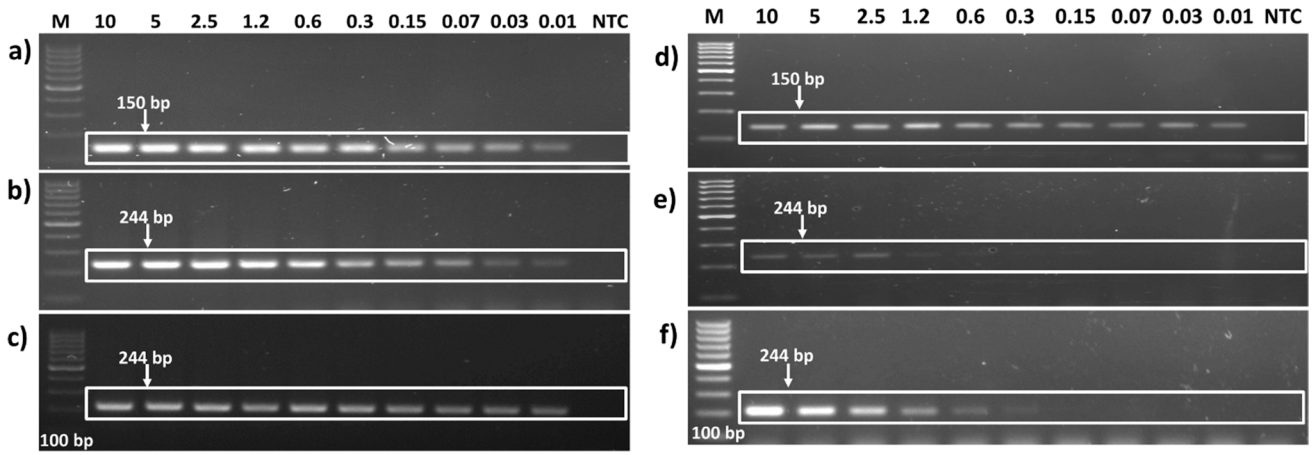

Fig. 2 Sensitivity assay with 10 ng to 0.01 ng DNA concentration using DNA extracted from leaves and dried fruit tissues with species-specific primers. **a, b, c** *Terminalia bellirica* (TB), *Terminalia chebula* (TC), and *Phyllanthus emblica* (PE) for the DNA extracted from the leaves of the respective 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

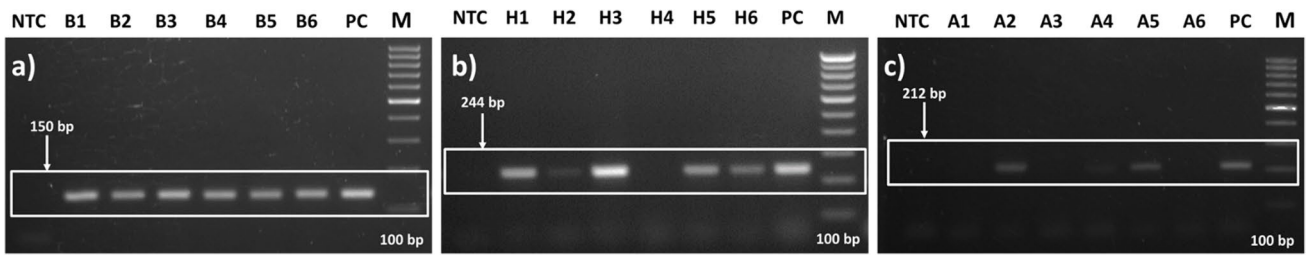

Fig. 3 Species-specific PCR assay with single drug market formulation with respective primers. **a** *Terminalia bellirica* (TB), B1 to B6 TB (Baheda) fruit powder, **b** *Terminalia chebula* (TC), H1 to H6 TC (Harde) fruit powder, and **c** *Phyllanthus emblica* (PE), A1 to A6 PE (Amla) fruit powder. 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

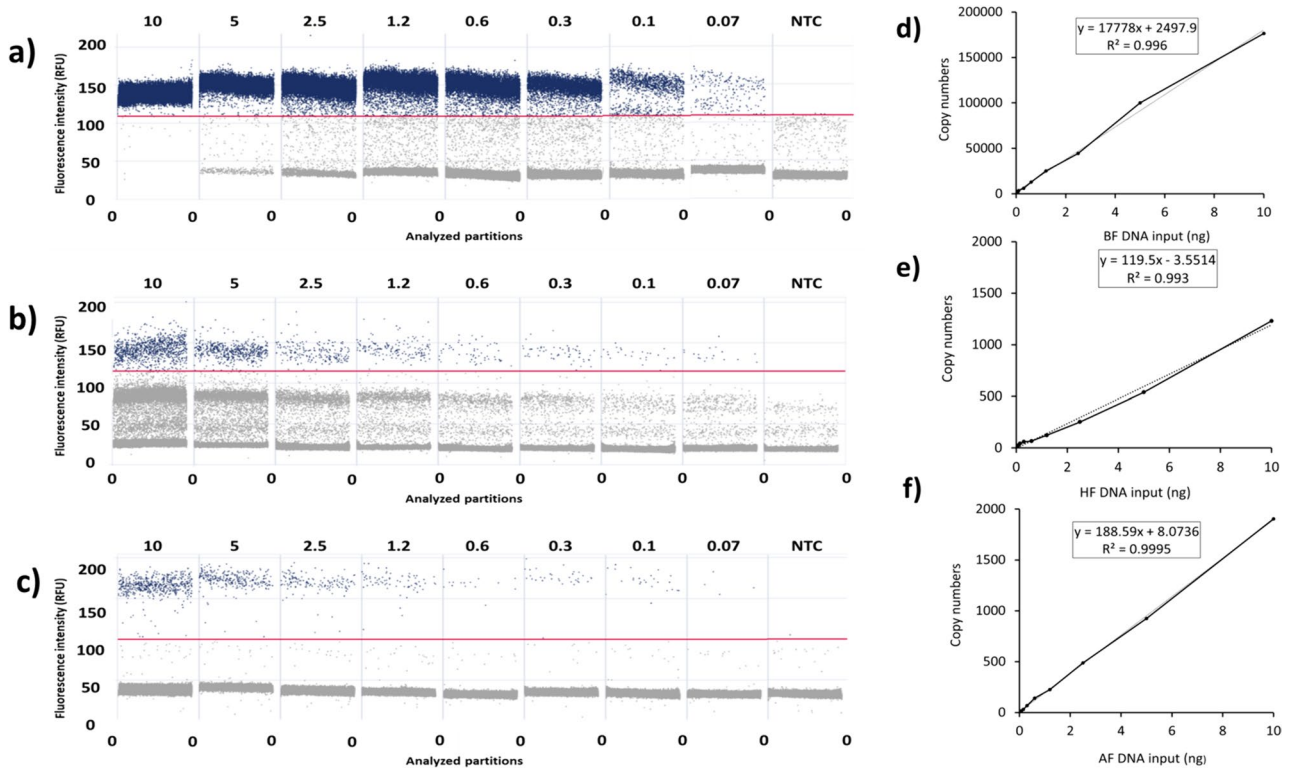

Fig. 4 Digital PCR assay with *Terminalia bellirica* (TB), *Terminalia chebula* (TC) and *Phyllanthus emblica* (PE) fruit DNA. The red line represents the fluorescence amplitude threshold. Positive partitions were observed above the threshold and negative partitions were below the threshold. NTC = no 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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Research Article

Advancing Herbal Product Authentication: A Comprehensive Review Of DNA-Based Approach For Quality Control And Safety Assurance

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ABSTRACT

The escalating popularity of herbal products in recent years has given rise to concerns about their quality and safety. The diverse origins of these products, rooted in local and traditional practices, pose challenges in defining and characterizing them, leading to varying regulatory frameworks and an influx of new dietary supplements and herbal medicines. This review explores the significance of DNA authentication methods, including species-specific PCR, DNA barcoding, metabarcoding, and digital PCR, in addressing the complexities of herbal product quality and safety. These techniques offer diverse applications, from rapid species identification to detecting trace contamination and quantifying adulterants. The review emphasizes the need for collaboration between regulatory authorities, industry stakeholders, and scientific communities to standardize and implement DNA-based authentication methods, establishing a new paradigm for ensuring authenticity and safety throughout the herbal product supply chain. The comprehensive examination of these DNA-based methods provides valuable insights into their role in safeguarding against contamination and adulteration while promoting sustainable sourcing practices in the herbal products industry.

Keywords: DNA authentication, species-specific PCR, DNA barcoding, metabarcoding, digital PCR, quality control and safety of herbal products

Introduction:

Herbal preparations have been integral to healthcare systems globally, with an increasing reliance on commercial herbal products in recent years (Raclariu-Manolică et al., 2023). However, the surge in popularity of these products has raised concerns regarding their quality and safety (Ekor, 2014). The diverse origins of herbal products, stemming from local and traditional practices, make defining and characterizing them challenging. This lack of consensus has led to varying regulatory frameworks and an influx of new dietary supplements and herbal medicines (Thakkar et al., 2020).

The terminology used to describe commercial herbal products includes herbal or botanical drugs, traditional or natural medicines, dietary supplements, and natural health products, each posing unique challenges for quality assessment (Heinrich et al., 2022; Shipkowski et al., 2018). As globalization expands the value chains of these products, concerns about responsible sourcing and sustainable supply chains have come to the forefront (Booker et al., 2012). While some herbal products classified as "medicines" undergo strict quality monitoring, "supplements" often face less rigorous regulations, potentially leading to safety issues (Posadzki et al., 2013).

Supply chain weaknesses, driven by high demand and increasing prices for herbal preparations, can result in accidental contamination or intentional adulteration for economic gain (Heinrich et al., 2022). Fraudulent operators may deceive conventional identification assays, substituting lower-cost plant species or adding non-plant materials to mimic the desired herbal product's chemical profile (Booker et al., 2012).

The application of taxonomic and analytical methodologies for the authentication of medicinal plants is widely recognized within the scientific community. The efficacy of analytical techniques in discriminating closely related plant species relies on the specific secondary metabolites present, a profile influenced by environmental variables, as well as variations in the processing and storage conditions of herbal formulations (Pant et al., 2021). However, for quality assurance and consumer safety, precise identification of the bioactive therapeutic components is paramount (Newmaster et al., 2013).

The necessity for universal authentication systems that can offer taxon identification with high confidence and the ability to differentiate among diverse plant species is evident. In the context of DNA-based authentication, DNA serves as a universal marker unaffected by tissue characteristics, age, environmental conditions, and processing/storage variations (Ancuta Cristina Raclariu, Heinrich, et al., 2018a). Major pharmacopeias globally, including the Chinese Pharmacopoeia, United States Pharmacopeia, British Pharmacopoeia, Japanese Pharmacopeia, and Hong Kong Chinese Materia Medica, advocate the use of DNA-based authentication methods (Wu & Shaw, 2022).

Various techniques such as Species-specific PCR assays (Li et al., 2021; Noh et al., 2021; Sharma & Shrivastava, 2016; Travadi, Sharma, et al., 2022a) and DNA barcoding (Balaji & Parani, 2022; Selvaraj et al., 2012; Vassou et al., 2016; Zahra, 2019) offer cost-effective means of identifying single or targeted plants. Concurrently, high-throughput sequencing-based metabarcoding (Frigerio et al., 2021; Pandit et al., 2021; Seethapathy et al., 2019; Yao et al., 2022) enables the simultaneous identification of multiple taxa within DNA mixtures extracted from herbal products. Consequently, researchers increasingly favor a multi-methodological or integrated analytical approach, combining both traditional analytical methods and advanced DNA-based techniques for enhanced medicinal plant authentication (Frigerio et al., 2021; Intharuksa et al., 2020; Travadi et al., 2023). DNA metabarcoding, while effective for identifying various components in a mixture, has a limitation in providing quantitative information about adulteration (Ancuta Cristina Raclariu, Heinrich, et al., 2018a). In contrast, Droplet Digital PCR (ddPCR) and Digital PCR (dPCR) are robust techniques that enable the detection and quantification of DNA from adulterants. These powerful methods offer the capability to precisely measure the abundance or concentration of specific DNA sequences, providing a quantitative aspect to the assessment of adulteration in a given sample (Travadi, Shah, et al., 2022; Yu et al., 2022a) (Figure1; Table 1).

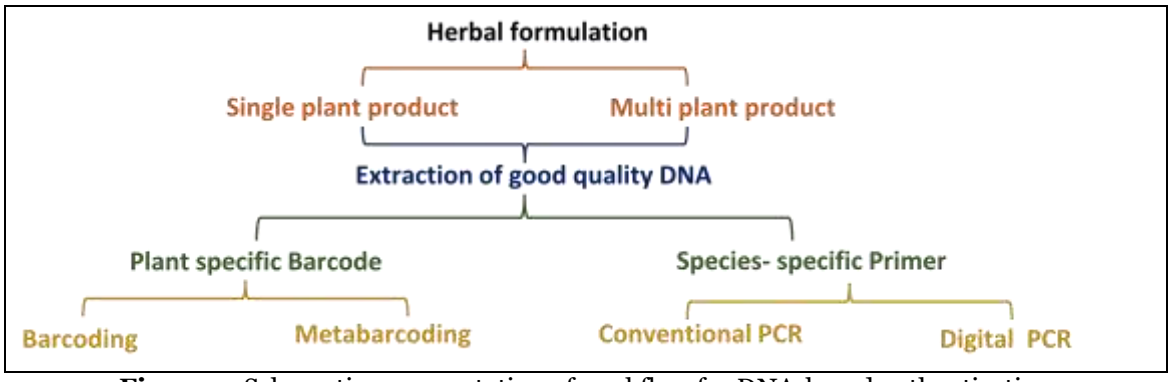

Figure 1. Schematic representation of workflow for DNA-based authentication

Table 1. Key points highlighting advantages and limitations of DNA-based authentication techniques for quality control in herbal products

DNA-based authentication technique	Advantage	Limitation
Species-specific PCR assay	• Rapid detection • Cost-effective • Determination of presence or absence of targeted species	• Inability to detect untargeted adulteration • Potential for non-specific amplification
Barcoding	• Sensitivity and specificity • Robustness • Resolution at Lower Taxonomy	• Inability to determine species in mixtures • Varying resolution power with different barcodes

Metabarcoding	• High throughput • Detection of Widespread Contamination in Mixtures	• Varying resolution power with different barcodes • High sensitivity for trace contamination • Lack of curated reference databases • PCR bias and primer fit compatibility • Not applicable for quantitative determination
Digital PCR	• High throughput • Detection of very low quantity of adulteration • Quantitative determination	• Lower dynamic range • Inability to determine untargeted adulteration

This review explores the significance of DNA authentication methods, specifically Species-Specific assay, DNA barcoding, metabarcoding, and dPCR, in addressing the complexities associated with herbal product quality and safety. It highlights their role in identifying and authenticating botanicals, safeguarding against contamination and adulteration, and promoting sustainable sourcing practices within the herbal products industry (Figure 2). Furthermore, the review emphasizes the need for collaboration between regulatory authorities, industry stakeholders, and scientific communities to standardize and implement DNA-based authentication methods. This collaborative effort can establish a new paradigm for ensuring the authenticity and safety of herbal products while fostering transparency and accountability throughout the supply chain (Figure 2; Table 2).

Table 2: Recent reports on applications of DNA-based authentication in herbal plant material

DNA-based authentication protocol	**Application on Plant material**	**Reference**
Species-specific PCR assay	*Panax ginseng, P. quinquefolius,* and *P. notoginseng* root materials	(Lu et al., 2022)
Multiplex-SCAR assay	Mistletoe species *Taxillus chinensis* and *Viscum coloratum* leaves and branch material	(Noh et al., 2021)
Multiplex-SCAR assay (chloroplast *trnH-psbA* intergenic spacer)	*Isatis indigotica* dried leaves powder	(Hsieh et al., 2021)
Duplex and Digital PCR assay	*Ocimum basilicum* and *Ocimum tenuiflorum* dried leaves powder	(Travadi, Sharma, et al., 2022)
Duplex and Digital PCR assay	*Piper nigrum* and *Carica papaya* dried berries	(Travadi, Shah, et al., 2022)
Species-specific PCR and Metabarcoding	*Bacopa monnieri* and *Centella asiatica* dried leaves powder	(Shah et al., 2023)
RbcL minibarcoding	Polyherbal formulation of Indian market	(Pandit et al., 2021)
ITS2 and *rbcL* metabarcoding	Single and Polyherbal formulation of Indian market	(Travadi et al., 2023)
qPCR and dPCR	*Actaea racemosa* (Black cohosh)	(Shanmughanandhan et al., 2021)
Droplet digital PCR	*Panax notoginseng*	(Yu et al., 2022)
ITS2 and *rbcL* metabarcoding	39 Thai herbal products listed on the Thai National List of Essential Medicines	(Urumarudappa et al., 2020)

		(NLEM)	
psbA-trnH and metabarcoding	ITS2	15 Herbal Teas	(Frigerio et al., 2021)
ITS2 metabarcoding		62 products, containing basil, oregano, and paprika collected from different retailers and importers in Norway.	(Ancuţa Cristina Raclariu-Manolică et al., 2021)
ITS2 metabarcoding		18 Milk thistle botanical formulations (teas, capsules, tablets, emulsion)	(Ancuţa Cristina Raclariu-Manolică et al., 2023)
ITS2 metabarcoding		71 herbal medicinal products were randomly purchased from Greek markets	(Anthoons et al., 2021)
psbA-trnH metabarcoding		3 Hedyotis herbal products collected from China and Thailand	(Yik et al., 2021)
ITS2, *mat*K, *rbc*L and *trn*H-*psb*A barcoding		DNA barcode library of plants listed in the Thai Herbal Pharmacopoeia (THP) and Monographs of Selected Thai Materia Medica (TMM) for 101 plant species	(Urumarudappa et al., 2022)
ITS and *matK*		Six *Momordica* species	(Kumar et al., 2020)
ITS2 barcoding and metabarocding		30 raw material samples, 10 food products and 12 herbal products	(Zhang et al., 2020)
ITS2 barcoding		52 Licorice products from Chinese market	(Li et al., 2023)
ITS2 and *trnL* metabarcoding		4 TCM preparations from Chinese market	(Yao et al., 2022)

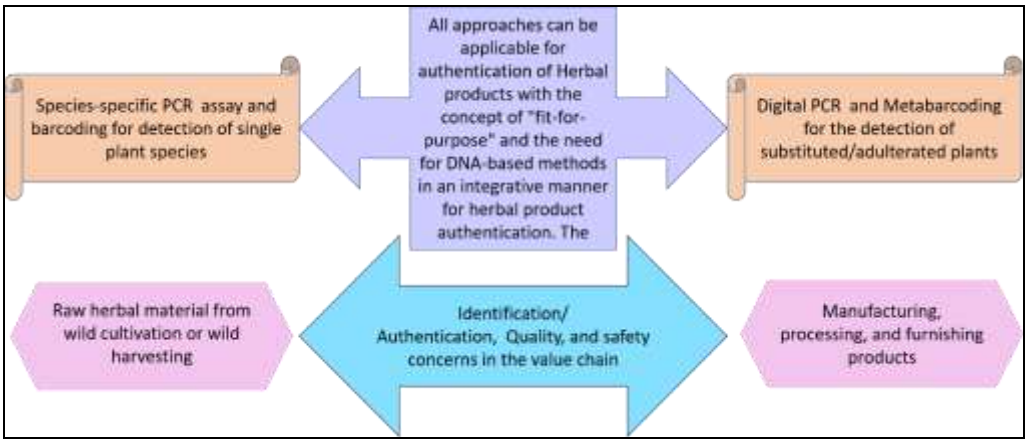

Figure 2. Utilizing DNA-based methodologies for the quality assessment and monitoring of herbal preparations in the value chain

Rapid and cost-effective Species-specific assay

Species-specific PCR assays play a pontial role in the authentication of herbal products, providing a rapid, accurate, and cost-effective means of identifying specific plant species within complex herbal formulation (Wu & Shaw, 2022). The development of these assays involves the use of species-specific primers derived from various sources such as Random Amplified Polymorphic DNA (RAPD)(Dnyaneshwar et al., 2006), Sequence Characterized Amplified Region (SCAR)(Dhanya et al., 2009; Kim et al., 2019; Shah et al., 2023), Inter-Simple Sequence Repeat (ISSR)(Kumar et al., 2018) or multiple sequence alignments (MSA) utilizing chloroplast genomes(Travadi, Shah, et al., 2022; Travadi, Sharma, et al., 2022b) and various barcode combination(Sharma et al., 2017). Chloroplast genome DNA barcode regions increase the probabilities to detect in compare to single locus barcode region due to sheared DNA in process herbal product, have been

increasingly employed for the development of species-specific primers(You et al., 2021). Zhang et al. (2017) demonstrated the use of chloroplast genome DNA sequences for the identification of *Echinacea* species (Zhang et al., 2017), and He et al. (2017) illustrated their application for barcoding *Lonicera japonica* (He et al., 2017). The development of specific primers for the detection of *Carica papaya* adulteration in Piper nigrum products and for Ocimum sanctum and Ocimum basilicum in Tulsi products has been instrumental in enhancing the authentication of botanicals (Travadi, Shah, et al., 2022; Travadi, Sharma, et al., 2022b). The criteria considered, such as high copy number of chloroplast DNA, short amplicon length, specificity, and sensitivity, have significantly contributed to the effectiveness of these primers in accurately identifying and differentiating the target botanicals within herbal products. In the context of lower taxonomic level identification, the utilization of Species-specific Primers derived from the Internal Transcribed Spacer (ITS) region has proven to be a more effective alternative for detections (Hsieh et al., 2021; Sharma et al., 2017). British pharmacies have been pioneers in adopting trnH-psbA-derived species-specific primers for the authentication of *Ocimum tenuiflorum* (Cartwright, 2016). Similar studies have been conducted on *Terminalia arjuna* (Sharma et al., 2017), *Senna tora* (Seethapathy et al., 2015), *Portulaca oleracea* (Xu et al., 2023), *Viscum coloratum* (Noh et al., 2021), *Aristolochia species* (Doganay-Knapp et al., 2018), and *Isatidis Folium* (Hsieh et al., 2021). These investigations contribute to the expanding scientific knowledge and emphasize the critical importance of rigorous authentication protocols in maintaining the quality control of herbal products.

Species-specific DNA markers, developed based offer high specificity, sensitivity, and applicability to single or multi-ingredient formulations. They are robust, rapid, and cost-effective, making them suitable for standard techniques in evaluation of raw material in value chain. However, it is important to note that species-specific assays are limited to detecting known target species and cannot identify unknown samples or unexpected contaminants. Sequencing-based identification, such as DNA barcoding, complements these assays by providing a broader scope for detecting known and unknown species. Overall, species-specific PCR assays are valuable tools in the authentication of herbal products, contributing to the overall quality and reliability of the herbal industry.

Advancements in DNA Barcoding for Herbal Products

DNA barcoding is a technique that uses a short, standardized fragment of the genome known as a "DNA barcode" to identify species (Hebert et al., 2003). This brief sequence of nucleotides can be derived from the chloroplast, mitochondrial, or nuclear genome and enables the identification of organisms at the species level. As such, DNA barcoding is considered to be the most effective method for species-level resolution and taxonomic identification(Little, 2014a). The process typically involves DNA extraction, polymerase chain reaction, sequencing, and sequence analysis. A comprehensive review analysed 17 potential barcode regions (*matK, rbcL, ITS1, ITS2 psbA-trnH ,atpF-atpH , ycf5,p sbKI,nad1,trnL-F,rpoB,rpoC1 atpF-atpH,and rps16*) extensively used in authenticating and identifying medicinal plants(Techen et al., 2014).

Global-level online data analysis was conducted to detect adulterants in herbal products using DNA barcoding. Adulteration rates were highest in Australia (79%), followed by South America (67%), Europe(47%), North America(33%), Africa(27%). Asia had the lowest percentage with Brazil being the highest distantly followed by Taiwan India USA Malaysia Japan South Korea Thailand China (Ichim, 2019). DNA barcoding has been successfully used in a range of studies to detect adulteration in products derived from *Ginkgo biloba* (Little, 2014b), *Actaea racemos* (Baker et al., 2012), *Senna,* and (Seethapathy et al., 2015). It has also been applied to identify common adulterants of endangered species such as genus Panax, and to recognize species with high toxic or allergenic potential found in herbal products(Wallace et al., 2012). Several studies have demonstrated the effectiveness of DNA barcoding in herbal product authentication and identification (Balaji & Parani, 2022; Vassou et al., 2016). These studies have shown that DNA barcoding can accurately distinguish between different species of plants used in herbal products, allowing for quality control and preventing mislabelling and adulteration.

Based on the extensive studies carried out with 7 barcode regions (*psbA-trnH, matK, rbcL, rpoC1, ycf5, ITS2 and ITS*), DNA barcoding has shown promising potential for authenticating and identifying medicinal plants. These regions have been used to study 8557 specimens from 5905 species belonging to 1010 diverse genera of 219 families in 7 phyla(Chen et al., 2010). The results have also led to the recommendation of using ITS2 as a potential barcode, with the ability to discriminate species up to 92.7% efficiency. Additionally, the creation of an API reference DNA Barcode library for 374 medicinal plants with the rbcL barcode has been instrumental. This library covers species from 308 genera, 112 families, and 45 orders (Vassou et al., 2016). Furthermore, the testing of 100 raw herbal products using the rbcL barcode revealed that 21% of the products were reported to contain adulterants (Vassou et al., 2016). Notably, DNA barcoding has made its debut in the British Pharmacopeia for the authentication of *Ocimum tenuiflorum*(Cartwright, 2016). This marks a significant step in the integration of DNA barcoding for the authentication of herbal products.

Despite its promising potential for authenticating and identifying herbal products, DNA barcoding encounters several limitations that require careful consideration. The extraction of DNA from processed herbal products proves challenging due to their composition, including bioactive secondary compounds, salts, polyphenols and polysaccaride, which readily interact with DNA, hindering the isolation process(Ancuta

Cristina Raclariu, Heinrich, et al., 2018a). To overcome these challenges, substances like bioactive charcoal, PVP, PVPP, and compounds resembling ascorbic acid are employed to remove secondary metabolites (Schenk et al., 2023). The physical processing of herbal products, such as boiling and grinding, further complicates the isolation process, resulting in sheared DNA, making it difficult to amplify barcodes like MatK(Parveen et al., 2016).

The extensive inter- and intra-species divergence in plants, coupled with consensus regions in DNA sequences, poses a challenge in identifying a universal barcode for diverse taxonomic studies. Unlike animals, selecting a single applicable barcode across various studies of plant diversity and taxonomic identification is challenging, given that the resolution and discrimination ability of a DNA barcode are directly proportional to taxonomic and environmental differences(Hollingsworth et al., 2011; Mishra et al., 2016a).

Moreover, DNA is omnipresent in every plant cell, regardless of its function. Consequently, DNA barcoding may not effectively detect specific bioactive compounds present in herbal products. For instance, in *Withania somnifera*, where the bioactive compound withanoid is primarily found in the roots, reported adulterants may involve other plant parts like stems of the same plant (Mundkinajeddu et al., 2014).

Despite these challenges, DNA barcoding remains a trending method for identifying medicinal plants and raw materials due to its resolving power. However, the analysis of DNA barcoding results in herbal products faces multiple challenges, including limited reference databases, inconsistency in the DNA barcode regions used for identification, and the potential presence of contaminants or fillers that can interfere with result accuracy(Mishra et al., 2016b; Parveen et al., 2016; Ancuta Cristina Raclariu, Heinrich, et al., 2018b). These challenges underscore the need for standardized protocols and comprehensive reference databases to enhance the reliability of DNA barcoding in herbal product authentication. In conclusion, while DNA barcoding holds great promise for authenticating and identifying herbal products, addressing these limitations is crucial for advancing the field. Continued research and innovative strategies are essential to overcome these challenges and improve the accuracy and reliability of DNA barcoding in the authentication of herbal products.

High -throughput Metabarcoding application for herbal products

Metabarcoding, a fusion of Next Generation Sequencing (NGS) and DNA barcoding, emerges as a powerful tool for identifying multiple taxa within a given sample, particularly in the realm of herbal products. The workflow involves DNA isolation, PCR amplification, adaptor ligation, library preparation, emulsion PCR, and NGS for bench work, accompanied by a bioinformatics pipeline encompassing sequence trimming, clustering, and BLAST analysis (Coghlan et al., 2012). This methodology combines the strengths of NGS and barcoding, overcoming the challenges of using a single plant barcode for species-level identification due to the plant kingdom's diversity, slow molecular evolution, and frequent cross-pollinations and hybridizations(Fazekas et al., 2009).

Several studies have demonstrated the efficacy of metabarcoding for the authentication of herbal products. Multi-barcode approaches, such as *ITS2* and *trnL*, have been employed to precisely identify plant species in various Chinese medicine and herbal teas (Frigerio et al., 2021; Xin et al., 2018). However, these studies also underscored limitations, including variability in barcode universality and resolution power, the absence of curated databases, and challenges in bioinformatics pipelines (Frigerio et al., 2021; Xin et al., 2018) .Metabarcoding has shown excellent outcomes in various studies for the authentication of herbal products. In these studies, results reveled that, labeled species in single drugs of *Echinacea* species (Ancuta Cristina Raclariu, Ţebrencu, et al., 2018), *Hypericum perforatum* (Ancuta Cristina Raclariu et al., 2017), and *Veronica officinalis* (Ancuta C Raclariu et al., 2017). Ivanova et al. (2016) identified key ingredient DNA in 8 out of 15 herbal products using the *ITS2* barcode via NGS(Ivanova et al., 2016). Cheng et al. (2014) demonstrated high quality in 27 tested herbal products with a high degree of non-filler and non-listed moieties (Cheng et al., 2014).

In India, where the market for herbal products is rapidly growing, the application of metabarcoding to detect raw plant materials in herbal medicine is not yet well-established. However, the sensitivity of DNA metabarcoding makes it a valuable method for detecting trace amounts of contamination, including pollen and other plant species introduced during cultivation, transport, and production (Pandit et al., 2021; Seethapathy et al., 2019; Shah et al., 2023; Travadi et al., 2023). Despite its sensitivity, DNA metabarcoding is not without challenges. Variables such as DNA quality and quantity, PCR amplification bias, library preparation, sequencing platform, and data analysis parameters can influence the results. Non-curated databases like NCBI GenBank may lead to incorrect identifications at lower taxonomic levels, emphasizing the need for further refinement in reference databases (Arulandhu et al., 2017; Pawluczyk et al., 2015; Staats et al., 2016).

In conclusion, DNA metabarcoding presents a robust approach for herbal product authentication, offering sensitivity to trace contamination. However, addressing challenges related to barcode universality, database curation, and bioinformatics pipelines is crucial for advancing the reliability and accuracy of metabarcoding in the herbal product authentication landscape. Ongoing research in screening new barcodes and variable regions within existing barcodes is essential to enhance the authentication of herbal products.

Exploring dynamics of digital PCR in herbal products

Digital PCR (dPCR) has emerged as a powerful technology for detecting and quantifying DNA or RNA molecules in various applications, including herbal product authentication. The technique involves partitioning a sample into thousands of nanoliter-sized droplets or partitions, enabling the accurate detection and quantification of low-level target DNA, even in complex samples with inhibitors(Hindson et al., 2011). Compared to quantitative PCR (qPCR), dPCR offers higher sensitivity, precision, and the advantage of providing absolute measures of nucleic acid concentration without the need for standard curve (Dingle et al., 2013). This capability makes dPCR a robust tool for authenticating herbal products where DNA quality and quantity can be compromised due to secondary metabolites present in plant material(Morcia et al., 2020).

In the study conducted, a dPCR assay efficiently detected 1.0% (w/w) papaya adulteration in Piper products, showcasing its sensitivity (Travadi, Shah, et al., 2022). Similar studies by Yu et al. (2021a) and You et al. (2021) demonstrated the ability of droplet dPCR to detect 1% adulteration in *P. notoginseng* and *canola honey*, respectively (You et al., 2021; Yu et al., 2022b).

In conclusion, dPCR stands out as a valuable tool for herbal product authentication, offering high sensitivity, specificity, and absolute quantitation capabilities. Its application in detecting adulteration and quantifying target DNA in complex mixtures contributes to the overall quality control of herbal products. Continued research and utilization of dPCR in conjunction with other molecular techniques are essential for advancing the authentication protocols in the herbal industry.

Conclusion

DNA authentication methods have proven to be highly effective in overcoming the intricate challenges associated with ensuring quality control and safety in the realm of herbal products. The holistic approach to quality control within this domain spans the entire spectrum of production, encompassing the procurement of raw materials through to the final product. In this complex landscape, DNA-based methodologies such as species-specific PCR, barcoding, metabarcoding, and Digital PCR have emerged as promising tools with distinct applicabilities. It is imperative to acknowledge that each of these techniques is characterized by specific technological prerequisites, cost considerations, and levels of expertise essential for the accurate assessment of herbal product quality or quantity. Each approach has defined applicability and inherent limitations, emphasizing the importance of judicious selection based on the specific context of analysis.

The diversity of methodologies highlights the growing consensus on the significance of adopting multiple or complementary authentication approaches. This strategic alignment with the "fit-for-purpose" principle is paramount in raising benchmarks for quality control and safety in herbal products. The integration of various complementary techniques allows practitioners to enhance the robustness and reliability of their assessments, effectively addressing dynamic challenges in the herbal product industry. This comprehensive approach is essential for meeting contemporary demands, ensuring the integrity and safety of herbal products, and seamlessly incorporating the latest scientific knowledge and technological advancements in this evolving field.

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Conflict of Interest

The authors have no conflict of interest

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