Journal of Medicinal Plants for Economic Development
ISSN: (Online) 2616-4809, (Print) 2519-559X

High-quality DNA extraction for *Desmodium gangeticum*: A medicinal shrub of Shivalik Himalayas

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Dates:
Received: 26 Sept. 2024
Accepted: 12 Nov. 2024
Published: 23 Jan. 2025

How to cite this article:
Singh, S., Bhandari, M.S. & Dwivedi, N., 2025, 'High-quality DNA extraction for *Desmodium gangeticum:* A medicinal shrub of Shivalik Himalayas', *Journal of Medicinal Plants for Economic Development* 9(1), a272. https://doi.org/10.4102/jomped.v9i1.272

Background: *Desmodium gangeticum*, a crucial medicinal shrub from the Himalayan Shivalik region, is an economically important species globally. It contains a vast range of secondary metabolites, majorly polyphenols, that interfere with high-quality deoxyribonucleic acid (DNA) extraction by binding their oxidised form to DNA covalently and rendering it unusable for molecular research.

Aim: This study was focused on developing a protocol for the isolation of high-quality DNA from *D. gangeticum*, which is essential for carrying out molecular biological experiments. Also, the isolation of DNA in purified form has its significance in facilitating precise genetic analysis such as sequencing, restriction digestion and polymerase chain reaction (PCR)-based trials.

Setting: This study was conducted under controlled laboratory environment at Manav Rachna Centre for Medicinal Plant Pathology, Department of Biotechnology, Manav Rachna International Institute of Research and Studies, Faridabad, India.

Methods: Advanced techniques, commercial kits and rigorous modifications in existing DNA extraction buffer conditions and extraction protocols from both fresh and dried leaves are considered to optimise high-quality DNA isolation.

Results: With the standardised buffer conditions and protocol, high-quality DNA with purity indicated by a 260/280 nm absorbance ratio in a range of 1.8 to 2.0 with 250 ng/μL – 950 ng/μL concentration from 0.5 g of leaf sample and also validated by agarose gel electrophoresis showing reduced contaminations and intact DNA was isolated.

Conclusion: This standardised buffer condition and protocol led to high-quality DNA isolation, which was found good for restriction digestion using endonuclease enzymes, PCR and other molecular biology techniques, highlighting the ongoing efforts to enhance the reliability of genetic studies in medicinal plants.

Contribution: This study adds to the existing knowledge about the strategies and procedures for extracting high-quality DNA and good yield from leaf samples of *D. gangeticum.*

Keywords: *Desmodium gangeticum*; DNA isolation; medicinal plant; restriction digestion; modified CTAB; secondary metabolite; polyphenols; PVPP.

Introduction

Desmodium gangeticum Linn. DC (Vern: Hindi-Shalparni, Sanskrit-Prishiparni) is one of the vital endangered ethnomedicinal shrubs extensively spread in tropical and subtropical environments of Shivalik hills and Gangetic plains of Northern India (Ahmad Hajiet al. 2016). The plant has been widely used in the traditional Indian system of medicine, either alone or in combination with other drugs (Vasani et al. 2022; Wink 2015), as a major ingredient in more than 68 ayurvedic formulations, highlighting its very high therapeutic value in Ayurveda (Amjad et al. 2017; Margret 2017). Beyond India, *D. gangeticum* is also popular worldwide. In countries such as Malaysia, Vietnam, Thailand and the Indo-China region, its root and leaf decoction is used against diarrhoea or as a sedative for children, as well as to treat coughs, swellings and kidney problems. Also, the roots are applied to the gums for toothache and the leaves for headache. In Uganda's Bulamogi community, the roots are chewed to cure premature ejaculation (Mohan et al. 2021; Basu et al. 2023). *Desmodium gangeticum*, a revered medicinal plant, contains a diverse range of secondary metabolites crucial for its therapeutic effects (Al-Khayri et al. 2022; Bildziukevich, Wimmerová & Wimmer 2023; Mishra et al. 2005). Genetic analysis of *D. gangeticum* is crucial because of its considerable

Note: Additional supporting information may be found in the online version of this article as Online Appendix 1.

medicinal importance and abundant phytochemical content. Understanding the genetic diversity within *D. gangeticum* populations is crucial for breeding programmes and conservation efforts, ensuring the preservation of valuable genetic resources (Heider et al. 2009; Nandanwar et al. 2017; Yan et al. 2020). Moreover, genetic analysis facilitates the elucidation of genes accountable for synthesising bioactive compounds endowed with therapeutic attributes in the plant.

For genetic studies and molecular research, the isolation of pure, intact and high-quality deoxyribonucleic acid (DNA) is essential for the integrity of DNA for error-free results (Tan & Yiap 2009; Sahare & Srinivasu 2012). High-quality DNA is indispensable in molecular biology because of its pivotal role in ensuring the accuracy and reliability of the experimental results across various techniques such as sequencing, restriction digestion, polymerase chain reaction (PCR), and its applications specifically in genetic analysis. Concurrently, stringent maintenance of DNA purity levels is imperative to forestall the presence of contaminants that might compromise the fidelity of sequencing reactions, encompassing extraneous proteins, salts and other undesirable entities. Hence, rigorous quality assurance protocols, commonly entailing spectrophotometric or fluorometric methodologies, are conventionally deployed to ascertain DNA yield and purity (Horne et al. 2004; Norman & Dinauer 2016; Irfan et al. 2013). Notably, complex pathways involved in secondary metabolite biosynthesis or stress response mechanisms may complicate DNA isolation procedures by introducing contaminants or inhibitors that interfere with DNA extraction and purification processes. Co-isolation of highly viscous polysaccharides and degradation of DNA because of endonucleases are two issues encountered in the isolation and purification of high molecular weight DNA from certain medicinal and aromatic plant species. Likewise, inhibitor compounds, polyphenols and other secondary metabolites either directly or indirectly interfere with enzymatic reactions (Loomis 1974; Weising et al. 1994).

Overcoming these challenges requires the development of specialised protocols and techniques tailored to specific plant species or tissues, including the application of purification techniques, enzymatic treatments, and improved extraction buffers. There are several methods for obtaining high-yield pure DNA from plants that have a large number of secondary metabolites (Peterson, Boehm & Stack 1997). Maintaining DNA integrity involves careful sample collection, storage and extraction, with assessment using spectrophotometry and gel electrophoresis, highlighting the critical nature of high-quality DNA in advancing scientific progress (Lucena-Aguilar et al. 2016). For genomic DNA isolation, fresh leaf samples are often advised by DNA extraction techniques; nevertheless, this appears to be impractical when the samples are obtained from uncommon or difficult-to-reach locations. Under these circumstances, this study employs an experimental methodology to evaluate and standardise DNA extraction methods, incorporating various buffers and commercial kits. Deoxyribonucleic acid samples collected from biological specimens are processed using optimised buffer solutions, each prepared meticulously and applied in conjunction with the protocols specified by selected DNA isolation kits. The extracted DNA undergoes purification steps post-extraction to remove contaminants, followed by assessment through agarose gel electrophoresis and spectrophotometric analyses to determine concentration, purity and quality. Finally, it formulates the strategies and procedures for extracting high-quality DNA and yield from fresh as well as dried leaf samples of *D. gangeticum.*

Material and methods

Leaf tissue sample collection and preparation

Thirteen fresh indigenous accessions of *D. gangeticum* were collected in September 2023 from natural populations belonging to different provenance(s) across the Bandhwari regions, positioned between 30°22′54.81″ latitude and 78°00′42.78″ longitude range of Uttarakhand, India (Online Appendix 1: Table 1-OA1). Samples were immediately dried up with silica gel and brought to the laboratory of the Agriculture Biotechnology Division, The Energy and Resources Institute, Gurugram, Haryana. After that, all samples were lyophilized and stored at –80 °C until extraction.

Standardised extraction buffer composition

A base extraction buffer: 50 mM Tris (hydroxymethyl) aminomethane (Tris buffer – pH 8.0), 1.4 M sodium chloride (NaCl), 10 mM ethylenediamine tetra-acetic acid (EDTA – pH 8.0), 2% cetyltrimethylammonium bromide (CTAB), 2 mM β-mercaptoethanol (BME) was used. The detailed listing of reagents is mentioned in Online Appendix 1. Afterwards, four distinct buffer solutions were formulated incorporating modifications involving CTAB, followed by the application of three distinct DNA isolation kits (EDNA plant DNA extraction kit, Molecular diagnostics [MDI] plant DNA isolation kit and Universal DNA isolation kit) to assess the comparative

TABLE 1: Composition of different buffer solution optimised for high quality DNA extraction in Desmodium gangeticum.

Buffer	Variable	Reagent 1	Reagent 2	Reagent 3	Reagent 4	Reagent 5
Buffer A	Components	Tris	NaCl	EDTA	BME	PVP
	Working concentration	100 mM	1.4 M	50 mM	2 mM	2%
Buffer B	Components	Tris	NaCl	EDTA	BME	SDS
	Working concentration	100 mM	1.4 M	50 mM	2 mM	2%
Buffer C	Components	Tris	NaCl	EDTA	BME	GuHCl
	Working concentration	100 mM	1.4 M	50 mM	2 mM	4 M
Buffer D	Components	Tris	NaCl	EDTA	BME	TritonX-100
	Working concentration	100 mM	1.4 M	50 mM	2 mM	1%

EDTA, Ethylenediamine tetra-acetic acid; BME, β-mercaptoethanol; PVP, polyvinylpyrrolidone; SDS, sodium dodecyl sulphate.

effectiveness of a commercial kit against the CTAB method for DNA isolation. These buffers were designated as Buffer A, Buffer B, Buffer C and Buffer D (Table 1). Out of all four buffers, Buffer A was further optimised for better results, and the composition of the final modified DNA extraction buffer is 100 mM Tris buffer (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 2% CTAB, 2.5% polyvinylpyrrolidone (PVP), 2 mM BME.

Standardised extraction and purification protocol

The procedure of DNA isolation and purification is as follows:

- 0.5 g plant samples (lyophilised dry tissue) were taken and grinded using liquid nitrogen in a mortar and pestle
- The fine powder was then transferred into a 2 mL Eppendorf tube, and 700 µL of preheated modified extraction buffer was added immediately.
- The tubes were inverted for proper mixing of reagents with the fine powder (leaf samples) and subsequently placed in a water bath to incubate for 45 min with inverted mixing in between
- Afterwards, an equal volume of chloroform:isoamyl alcohol (24:1) was added into the same tube
- Lysate was properly mixed by inverting the tube for at least 2 min, then centrifuged at 13 000 rpm at 23 °C for 10 min
- The aqueous phase was transferred without disturbing lower phases into a fresh 2 mL eppendrof tube, to which an equal volume of chloroform:isoamyl alcohol (24:1) was added afresh
- The components were mixed properly by inverting the tube for at least 2 min and centrifuging at 13 000 rpm at 23 °C for 10 min
- Again, the aqueous phase without disturbing the lower phases was transferred into a fresh 1.5 mL eppendrof tube, to which a double volume of pre-chilled isopropanol was added, mixed inverted, and then 1 volume of 5 M NaCl was added and mixed properly
- After mixing and incubating, the samples were kept at −20 °C for overnight precipitation
- The next day, the samples were centrifuged at 13 000 rpm at 4 °C for 10 min
- Subsequently, the DNA pellet was washed with 500 µL 70% ethanol and centrifuged again at 13 000 rpm at 23 °C for 3 min
- The washing step was repeated three times
- After washing, the samples were air-dried until the ethanol was completely evaporated
- The pellet was dissolved in 50 µL of 1 × Tris-EDTA (TE) buffer and rested for 5–10 min at room temperature, which was then treated with RNAase A
- Afterwards, 5 µL RNAase A was added into the tube and incubated it for 20 min at 37 °C.
- Finally, the isolated DNA was run and checked on 0.8% agarose gel in 0.5× Tris-Borate EDTA (TBE) buffer.

DNA quantification

Genomic DNA quantification was accomplished using a NanoDrop spectrophotometer, a device commonly used for this purpose because of its precision and minimal sample requirement. The concentration of DNA in each sample was determined using the formula: DNA concentration (ng/µL) = absorbance at A260 × dilution factor × conversion factor. Subsequently, the purity of the DNA in each sample was assessed by calculating the A260/A280 ratio. The concentration and purity values for each sample were then documented for further analysis.

Preparation of agarose gel electrophoresis

The analysis of DNA quality was conducted through agarose gel electrophoresis, employing a gel concentration of 0.8%. The electrophoresis process utilised a 0.5× TBE buffer, which included 2 µg/mL of EtBr and was conducted at a constant voltage of 80 V over 50 min. After electrophoresis, the DNA bands were visualised, and images were captured using an Azure C150 Gel Imaging System.

DNA digestion analysis

For the DNA digestion analysis, the process commenced by setting the conditions for the enzymatic digestion at a controlled temperature of 37 °C. This enzymatic reaction was allowed to proceed overnight, ensuring thorough digestion of the genomic DNA. The reaction was conducted in a total volume of 20 µL, carefully prepared to contain 1 µg of the genomic DNA under investigation. In addition, 2 µL of a specialised enzyme digestion buffer, 10× fast digest (FD) buffer, was added to optimise the reaction conditions. This buffer is formulated to enhance the activity and specificity of the restriction enzyme, thereby facilitating efficient DNA cleavage at specific recognition sites. The inclusion of these components in the reaction mixture ensured that the DNA digestion process was conducted under controlled and standardised conditions, allowing for precise and reproducible analysis of DNA fragments generated by the enzymatic cleavage.

Ethical considerations

This article followed all ethical standards for research without direct contact with human or animal subjects.

Results

The DNA isolation procedure employing diverse CTAB modifications yielded outcomes below anticipated standards, typified by discernible instances of DNA degradation, diminished yield and contamination. Spectrophotometric analysis unveiled absorbance spectra suggestive of the presence of impurities and nucleic acid fragmentation. Consequently, the evaluation of DNA concentration and purity was foregone owing to the compromised structural integrity evident in the isolated DNA specimens. The experimental approach involved a systematic exploration of various buffers and protocols, including modifications of the widely used CTAB method and the assessment of commercially available DNA

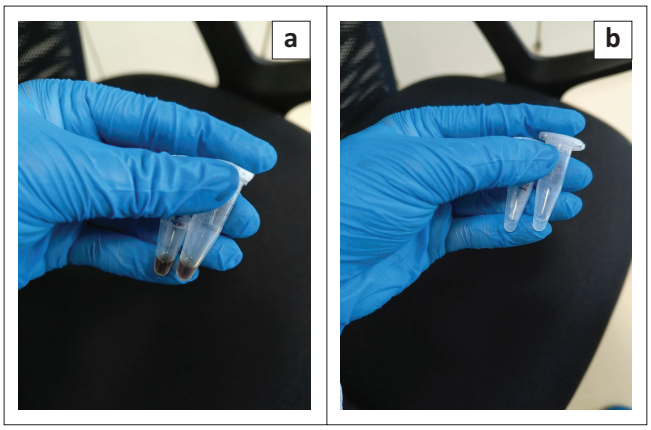


FIGURE 1: Deoxyribonucleic acid (DNA) observed after extraction through two distinct protocols in *D. gangeticum*: (a) brownish hue in the DNA extracted using Doyle and Doyle method and (b) clear transparent high-quality DNA through optimised protocol.

isolation kits. Each buffer and kit were thoroughly tested to evaluate its efficacy in yielding high-quality DNA suitable for molecular analyses. However, despite rigorous testing, the results revealed substantial challenges that underscored the complexity of DNA extraction from plant materials. Following a rigorous process involving numerous modifications and thorough analysis of the CTAB method alongside various commercial DNA isolation kits, a final standardised DNA isolation method tailored specifically for *D. gangeticum* was developed. This method was meticulously optimised to ensure optimal DNA yield, purity and integrity. *Desmodium gangeticum* possesses a huge number of polyphenols. During cell lyses, polyphenols come out of the vacuoles and are readily oxidised by cellular oxidases. The oxidised polyphenols undergo irreversible interactions with nucleic acids and cause enzymatic browning of the DNA pellet and shortening its maintenance time, rendering it unusable for molecular research (Katterman & Shattuck 1983; Twaij & Hasan 2022). With the previous isolation method, a dark brownish hue was observed, while with the standardised protocol, clear, transparent, high-quality DNA was obtained (Figure 1). Here, PVP and polyvinyl polypyrrolidone (PVPP) to prevent phenol oxidation during cell lysis were employed. These substances act as adsorbents, especially effective under acidic conditions. Polyvinyl pyrrolidone forms complexes with polyphenols through hydrogen bonding, separating polyphenols from DNA. Studies have found NaCl + CTAB is an effective composition. When the salt concentration increases (above 0.5 M NaCl), CTAB (cationic detergent) preferentially binds to polysaccharides, forming complexes that can be removed during subsequent steps such as chloroform extraction (Kurian et al. 2010) and with this the DNA remains in solution.

Notably, the inclusion of 2.5% PVP and 2 mM BME played a pivotal role in enhancing DNA extraction efficiency and preventing potential degradation; certainly, with lesser concentration, the DNA quality was improved with a light

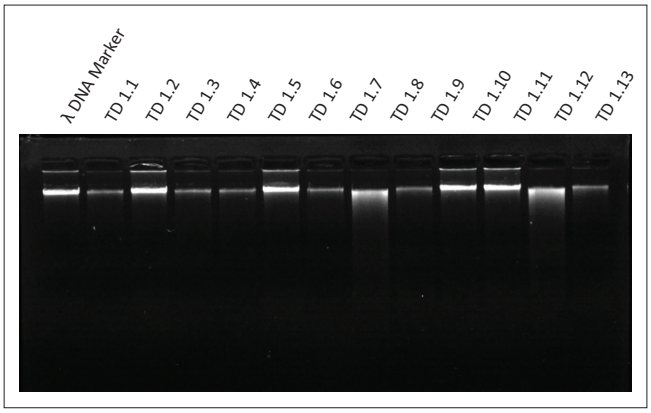


FIGURE 2: Agarose gel electrophoresis revealing extracted genomic deoxyribonucleic acid from 13 leaf samples of *D. gangeticum* using the optimised buffer method.

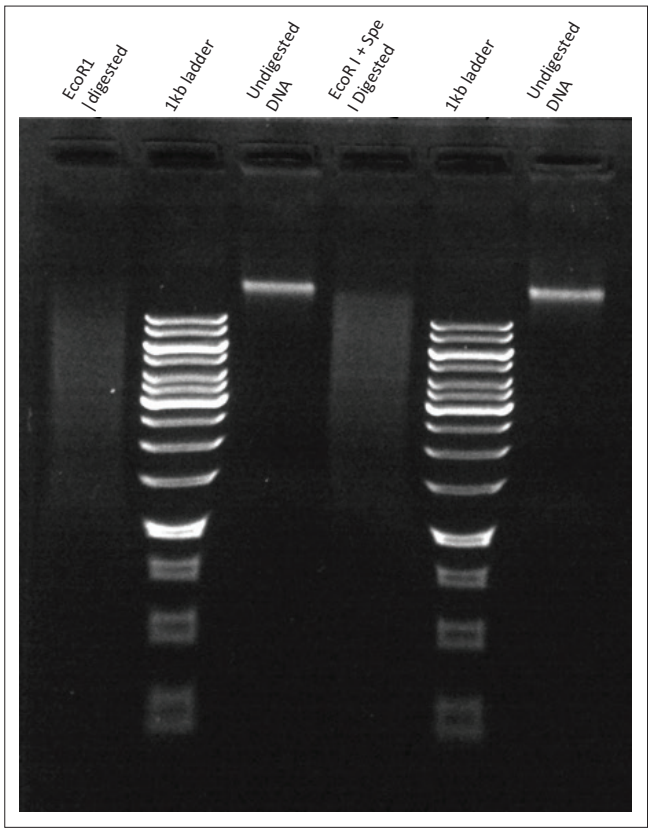
TABLE 2: Quantitative estimates of extracted deoxyribonucleic acid from leaf Samples of Desmodium gangeticum using DNA extraction protocol.

S. no.	Isolation method	DNA yield (ng µL^{-1})	DNA purity (260 280^{-1})
1	Original base buffer	35.2	1.189
2	Buffer A	126.2	1.46
3	Buffer B	54.9	1.56
4	Buffer C	65.5	1.33
5	Buffer D	41.4	1.39
6	EDNA Kit	40.7	1.66
7	Universal Kit	89.3	1.48
8	MDI Kit	62.1	1.24
9	Standardised buffer	795.4	1.95
10	Standardised buffer	268.7	1.90
11	Standardised buffer	657.4	1.82
12	Standardised buffer	455.8	2.15

DNA, deoxyribonucleic acid; S. no., serial number; ng µL, nanogram per microlitre.

brown colour, while with more concentration, the DNA isolated was observed to be degraded (Online Appendix 1: Figure 1-OA1). Additionally, a significant change was observed with the usage of a 5 M NaCl solution during overnight precipitation to facilitate DNA precipitation and purification. The standardised protocol-isolated DNA was checked on 0.8% agarose gel and visualised using a gel documentation machine. It was observed that the fine, intact and intense bands showed good quality of the extracted DNA (Figure 2). Furthermore, the nanodrop readings for extracted DNA were also promising in comparison to the previous method, showing an acceptable range of 260/280 along with a high concentration of DNA (Table 2). A 260/280 nm absorbance ratio from 1.8 to 2.0 was observed, and this signified that the DNA possessed high purity, suggesting the absence of proteins and phenols. To studyDNA sequence and carry out further DNA-based analyses for this species in our laboratory, two restriction endonuclease enzymes (EcoR I FD and Spe I high fidelity [HF]) were used for restriction digestion chosen according to available data sequence from NCBI GenBank.[1] EcoR I FD enzyme for single digestion and combination of EcoR I along with Spe I enzyme for double digestion were used and visualised on 0.8% agarose gel (Figure 3). It shows the promising result for restriction digestion of DNA as there is

1.Visit: https://www.ncbi.nlm.nih.gov/datasets/taxonomy/670324/.



Note: Digested products were separated on 0.8% agarose gel stained with ethidium bromide, in 0.5× TBE buffer. The digested products were visualised by UV fluorescence. Molecular marker 1 kb ladder was used (Lane 2 & 5), whereas undigested DNA was observed in (Lane 3 & 6).

FIGURE 3: Deoxyribonucleic acid (DNA) samples extracted by the standardised method and digested with EcoR I (Lane 1) and combination of Ecor I and Spe I (Lane 4) enzymes.

no amount of undigested DNA or other contaminating bands were observed in digested DNA in comparison to undigested DNA given same buffer and temperature conditions. This shows that the standardised protocol is useful for isolating contaminant-free DNA, which is required for any further DNA analyses.

Discussion

Medicinal plants such as *D. gangeticum* present challenges in the DNA extraction and purification processes because of the abundance of phytochemical compounds, polysaccharides, polyphenols, and other secondary metabolites in plant tissues that impede DNA isolation by binding to or co-precipitating with the DNA, thereby reducing the yield or quality of the extracted DNA (Sahu, Thangaraj & Kathiresan 2012; Venkatachalam & Muthukrishnan 2012). *Desmodium gangeticum* possesses a huge number of polyphenols. Polyphenolic compounds are particularly problematic because of their propensity to oxidise during the lysis of plant tissues (Joshi, Hakim & Patel 2023). Upon oxidation, polyphenols form quinones, which readily bind to nucleic acids and proteins. This binding not only co-precipitates with DNA during isolation but also causes extensive degradation and fragmentation of the DNA, severely affecting its yield and integrity (Kurian, Philip & Varghese 2005; Kurian et al. 2010; Loomis 1974). Standardising DNA isolation procedures is crucial for molecular biology research in order to ensure consistency, repeatability and reliability of results across different experiments and lab environments. Establishing a standardised protocol involves optimising various steps, including sample preparation, lysis, DNA extraction and purification, to achieve consistent yields and high-quality DNA. The optimisation of the DNA isolation process minimises the variability between samples and experiments, enabling more accurate comparisons and interpretations of data. The resulting DNA is frequently inappropriate for use in subsequent processes and of low purity. Secondary chemicals always make it more difficult to isolate DNA and perform any further reactions utilising DNA preparations such as PCR or restriction digestion. Restriction digestion is a versatile and indispensable tool in molecular biology that allows for precise manipulation, analysis and understanding of DNA sequences, and hence it is very much required to isolate high-quality DNA to perform restriction digestion, which leads to exploring new aspects of genetic studies. Since the inception of CTAB-based techniques for extracting DNA from plant leaves, efforts have been made to refine the method to mitigate the presence of contaminants such as polyphenols and polysaccharides inherent in plant tissues. In the pursuit of refined DNA isolation methodologies tailored to *D. gangeticum*, an exhaustive exploration of various buffers and protocols was undertaken. This endeavour aimed to optimise the DNA isolation process, ensuring robust and reproducible results essential for comprehensive genetic analyses.

The goal of the study was to develop a more sophisticated procedure that would allow for genetic analysis while clarifying the differences between two widely used approaches: commercial kits and appropriately modified versions of the CTAB method. Through systematic experimentation and comparative analysis, the efficacy of different buffers and protocols was diligently evaluated, with the overarching goal of standardising the DNA isolation procedure for *D. gangeticum*. The evaluation of four different DNA isolation buffers along with the examination of three commercial DNA isolation kits revealed critical insights into the efficacy and challenges associated with DNA extraction processes. Since its initial adoption, the CTAB method for extracting DNA from plant leaves has undergone numerous modifications to minimise contaminants such as polyphenols and polysaccharides found in plant tissues. While existing DNA extraction techniques effectively isolate DNA suitable for PCR amplification or restriction digestion, they involve lengthy incubations, several precipitation stages, and ethanol washes to obtain high-purity and ribonucleic acid (RNA)-free genomic DNA. These extra steps decrease the overall yield and result in lower quantities of high-quality DNA. The results of this study revealed that the quality of each DNA sample extracted was assessed using a NanoDrop spectrophotometer and agarose gel electrophoresis. The Nanodrops absorbance profile helps to identify contaminants such as proteins, salts and polysaccharides that may hinder DNA sequencing. In this

case, a 260/280 ratio of 1.8 suggested that the DNA was highly pure, free from proteins and polyphenols. To enhance DNA isolation and lessen the inhibitory effects of secondary metabolites such as phenolic compounds, polysaccharides and pigments, more PVP and concentrated NaCl were used during the DNA extraction process. Notably, PVP helps to prevent DNA damage by disrupting the interactions between DNA, RNA and phenolics, thereby enhancing the yield of DNA, and a high concentration of NaCl helps in salting out the contaminating compounds, other than these two other buffers and reagents (Online Appendix 1), such as β-ME, are used for denaturation of proteins for better extraction. Primary challenges observed included inadequate DNA yield or suboptimal PCR amplification outcomes. We faced challenges spanning from cell lysis through DNA extraction in the supernatant and subsequent reaction stages while adhering to the methodologies outlined by Doyle and Doyle (1990), as well as Murray and Thompson (1980). Similarly, adaptations to the conventional DNA isolation procedure involving Triton X-100, sodium dodecyl sulphate (SDS), guanidine hypochlorite and varied compositions of lysis and suspension buffers resulted in the development of a methodically standardised DNA isolation protocol. Finally, a standardised protocol with optimised conditions and salt concentrations was established for the isolation of high-quality DNA, which can be processed for any molecular biology technique such as restriction digestion or PCR for further analyses and genetic studies.

Conclusion

The study successfully developed an optimised protocol for the extraction of high-quality DNA from both fresh and dried leaves of *D. gangeticum,* a plant known for its medicinal properties and challenging content of secondary metabolites. Initial experiments with different buffers and commercial kits highlighted substantial difficulties, indicating the necessity for customised solutions to address contaminants such as polyphenols and polysaccharides. The enhanced CTAB-based method, which comprised 2.5% PVP, 2 mM BME and a 5 M NaCl solution for overnight precipitation, showed marked improvements in DNA yield, purity and integrity. By adjusting the extraction conditions and using specific reagents such as CTAB, PVP and NaCl in specific concentrations, the protocol overcame the inhibitory effects of compounds such as polyphenols and polysaccharides, which typically interfere with DNA isolation. These enhancements resulted in superior DNA quality, as verified by agarose gel electrophoresis and optimal absorbance ratios. The refined methodology not only improved the yield and purity of DNA but also facilitated more reliable genetic analysis through PCR and sequencing, highlighting the potential for enhancing molecular research and further genetic studies in medicinal species.

Acknowledgements

The corresponding author is grateful to National Medicinal Plant Board (NMPB), New Delhi India, for providing financial support (File No: Z.18017/187/CSS/R&D/DL-01/2022-23-NMPB-IV A) dated 01 August 2023.

Competing interests

The author reported that they received funding from National Medicinal Plant Board (NMPB), New Delhi, India which may be affected by the research reported in the enclosed publication. The author has disclosed those interests fully and has implemented an approved plan for managing any potential conflicts arising from their involvement. The terms of these funding arrangements have been reviewed and approved by the affiliated university in accordance with its policy on objectivity in research. The authors declare that they have no other financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Authors' contributions

S.S., was responsible for the conceptualisation, data curation, formal analysis, investigation, methodology, software, validation, writing – original draft. M.S.B., was responsible for the project administration, resources, visualisation, writing – review and editing. N.D., was responsible for the conceptualisation, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualisation, writing – original draft, writing – review and editing.

Funding information

National Medicinal Plant Board (NMPB), New Delhi India, for providing financial support (File No: Z.18017/187/CSS/R&D/DL-01/2022-23-NMPB-IV A).

Data availability

The data underlying this article are available in the article and Online Appendix 1.

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