An improved and effective DNA extraction protocol for *Pyracantha crenulata* with optimal PCR reliability

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Abstract

There are many challenges involved in obtaining high-quality DNA from tissues/ cells, especially when using plants as the raw material. Three different DNA extraction protocols were employed in order to isolate high quality of genomic DNA from *Pyracantha crenulata* leaves. *P. crenulata* is a complex, versatile, and evergreen shrub species in Rosaceae family which is ecologically, economically and culturally important. This species produces valuable antioxidants like polyphenols, polysaccharides and secondary metabolites which interfere with DNA extraction thus making molecular marker based studies difficult in this species. This study aimed to develop a simple, rapid, cost-effective and highly efficient protocol for *P. crenulata* leaves rich in salts and phenols. The results demonstrated that modified CTAB (double buffer) protocol is very effective in overcoming the challenges that could impede next-generation sequencing analysis. The yield of the extracted DNA was excellent ranging from 368 to 410 ng/ µL DNA with A260/280 ratio ranging from 1.80 to 1.84. The extracted DNA was amenable to PCR amplification making it suitable for DNA-based molecular marker studies. None procedures have been published for the purpose up this species, therefore, double buffer based extraction protocol is reported for the first time in *P. crenulata* and might prove useful in other species of family Rosaceae. This study describes an effective DNA isolation method that is appropriate for frequent population genetic screening. In conclusion, the double buffer protocol showed the most efficient and effective approach for extracting DNA from phenol rich, fresh, mature, and dry leaves.

Keywords: *DNA isolation, Hawthorn, ISSR markers, Polymerase chain reaction, Polyphenolic compounds, Underutilized species*

Introduction

Pyracantha crenulata (D. Don) M. Roem (Vern: Indian Hawthorn) is a complex species of the Rosaceae family in *Pyracantha* genus which contains three species viz., *P. loureiroi*, *P. crenulata* and *P. fortunea* (Feng et al., 2021). There are still taxonomic problems within the genus, with the identity of the *P. crenulata* complex being ambiguous when referring to the existing dichotomous species key. In the past, *Pyracantha* was treated as *Mespilus Linnaeus* (1753: 478) (Medikus and Kasimir, 1793; Persoon, 1806; Roxburgh and William, 1814) and *Crataegus* Linnaeus (1753: 478) (Spach, 1834). In the meantime, however, botanists and plant taxonomists have recognized *Pyracantha* as a separate genus on the basis of morphological and molecular findings (Lindley, 1845; Campbell, 1995, 2007; Eriksson, 2003; Kalkman, 2004; Potter, 2007). A total of nine species were included in the genus *Pyracantha* (Phipps, 1990). Feng et al. (2021) concluded that the *P. crenulata* complex varies enormously and continuously in terms of leaf blade characteristics (margin, tips, shape, length, width and broadest point). Therefore, these characteristics cannot be used to justify the subdivision of this complex. Since the unclear identification, it becomes very important to study this complex species at genetic level. *P. crenulata* is an evergreen, perennial, thorny and woody shrub species which has high ecological, economic and cultural values (Osmaston, 1927; Kavidayal and Uniyal, 2020). A high

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quality of DNA is crucial for the genetic studies of forestry species. Plant species generally produce secondary metabolites with antioxidant capacity to protect themselves from other organisms or stressful conditions (Cunill-Flores et al., 2023). Leaves of *P. crenulata* contain a large amount of polysaccharides, secondary metabolites and polyphenolic compounds (Guglani et al., 2022; Kewlani et al., 2023) due to which it is over-exploited by the local people and industrialists for making medicines (Shah et al., 2006; Singh et al., 2018; Pal et al., 2013). The plant is used in Ayurvedic medicine and is commercially harvested in forest areas for various medicinal preparations such as infusions from the dried fruits, liquid extracts and tinctures. Conventionally, this plant is used by the local people for fencing agricultural fields, making tool handles for agricultural implements, walking sticks and as fuel and for preparing herbal tea (Singh et al., 2018; Kunkel, 1984). The fruits of *P. crenulata* are traditionally consumed by locals in the northwestern Himalayan states of India to treat ailments caused by oxidative stress (Pal et al., 2013).

Molecular approaches using DNA sequencing and genotyping are essential for molecular characterization of the species to understand its population genetics which could further help in breeding and conservation programs. The foremost requirement in these studies is obtaining high quality DNA from the target species. The study and molecular analysis of plant genetic resources has been the subject of ongoing breakthroughs in the field of plant molecular biology. Each new discovery expands the boundaries of knowledge, and the sequencing is the most recent major success. However, purified genomic DNA, which is often required for many applications in molecular genetic studies, is much more difficult to obtain from trees than from other plants (Shepherd et al., 2002). Since the foliage and other tissues of trees often contain varying amounts of tannins, polyphenols and polysaccharides, these contaminants co-extract with the DNA, causing serious problems in obtaining genomic DNA. Such impurities also interfere with further DNA analysis (Sharma et al., 2018). Polysaccharides inhibit the digestion of restriction enzymes and the activity of *Taq DNA* polymerase (Demeke and Adams, 1992; Fang et al., 1992; Scott and Playford, 1996). Polysaccharides often co-precipitate with the extracted DNA, resulting in the formation of a highly viscous solution (Do and Adams, 1991). The DNA is unsuitable for restriction and Southern hybridization and often remains in the wells during electrophoresis. Consequently, many trees require highly complex extraction methods than other annual plants (Scott and Playford, 1996). There are several methods for isolating genomic DNA from plants that are currently being developed. However, it is unlikely that a single isolation method will be successful for different plants (Loomis, 1974). Also, the chemotypic heterogeneity of plant samples would not allow optimal yield with a single protocol (Weising et al., 1995), hence specific protocols must be followed for different plants.

The phytochemical contents present in the *P. crenulata* leaves including phenols, flavonoids, anthocyanins, β-carotene, lycopene etc. (Sharifi-Rad, 2020) can be problematic in DNA-based investigations. Considering this complexity, we have modified the reagent concentrations which are crucial for the genomic DNA extraction from the leaves of *P. crenulata* complex. The main impediments to DNA isolation are the presence of RNA, polysaccharides, proteins, polyphenols, and secondary metabolites. DNA extraction from *P. crenulata* is difficult due to the high content of secondary metabolites which interfere with the DNA extraction process. The DNA thus obtained is of poor quality, and the impurities complicate further DNA processing by interfering with the polymerase chain reaction (PCR) by inhibiting *Taq* polymerase. Therefore, the study aimed to develop an efficient protocol for extracting good quality of DNA from *P. crenulata* and to validate the standardized DNA extraction protocol by using inter-simple sequence repeat (ISSR) markers for optimizing PCR reliability which will help for conducting DNA-based molecular marker studies in this species.

Materials and methods

Study area and Sample Collection

Young leaves of *P. crenulata* were collected from Bhatta, Mussoorie (30°25′59.92″ N, 78°4′53.52″ E; 1608 m amsl) during the flowering season (April, 2022). The leaf samples were collected and instantly desiccated using silica gel and stored in deep freezer (-80°C) till further use. The experiment was conducted in Genetics and Tree Improvement Division, Forest Research Institute, Dehradun (Uttarakhand), India.

Genomic DNA Extraction

Initially, the conventional CTAB protocol (CCP) by Doyle and Doyle (1987) were used for *P. crenulata* DNA extraction. The modified activated charcoal protocol (ACP) described by the Križman et al. (2006) and the double buffer protocol (DBP) were attempted. The major modifications were employed in activated charcoal and double buffer protocols (Table 1, 2).

Activated Charcoal Protocol with modifications

The sterilized leaf sample (0.50 gm) was ground to a fine powder in liquid nitrogen and transferred to a 2-mL micro-centrifuge tube containing pre-warmed (60°C) charcoal buffer (1 mL) and incubated for 1 hour in a water bath at 60°C with occasional gentle swirling. The amount of 500 µL of Chloroform: Isoamyl alcohol (24:1) was added to the tubes and gently inverted to form a homogeneous emulsion. Then the tubes were centrifuged (Eppendorf, Hamburg, Germany) at 20,817 × g for 6 min at room temperature (25°C). The upper aqueous phase was transferred to a 1.5-mL micro-centrifuge tube and an equal volume (1:1) of chilled isopropanol (-20°C) was added to the supernatant. The tubes were carefully inverted until DNA filaments were visible and incubated overnight at -20°C to enhance precipitation. The tubes were centrifuged at 20,817 × g for 15 min at 4°C and the supernatant was discarded. The pellet was washed with 96 % ethanol (995 µL) and 3 M sodium acetate (5 µL) for 45 min. The tubes were centrifuged at 20,817 × g for 5 min at 4°C, and the supernatant was discarded. The pellet

Table 1
Chemical composition of extraction buffers used in different protocols (pH=8.0)

CCP	ACP	DBP	
		Lysis Buffer (LB)	Extraction Buffer (EB)
100mM Tris base	100mM Tris base	100mM Tris base	100mM Tris base
20mM EDTA	20mM EDTA	20mM EDTA	20mM EDTA
1.42 M NaCl	2M NaCl	1.42 M NaCl	1.42 M NaCl
5mM Ascorbic Acid	-	5mM Ascorbic Acid	5mM Ascorbic Acid
2 % PVP-40 (w/v)	2 % PVP-40 (w/v)	3 % PVP-40 (w/v)	3 % PVP-40 (w/v)
2 % CTAB (w/v)	2 % CTAB (w/v)	-	3 % CTAB (w/v)
-	0.5 % Activated Charcoal (w/v)	-	-
0.2 % β-ME (v/v)	-	0.5 % β-ME (v/v)	0.5 % β-ME (v/v)

Table 2
Modifications employed in three DNA extraction protocols

Protocol Used	Modification	Additional Steps
CCP	● None	● None
ACP	● Used 2 % PVP-40 for the removal of phenolic compounds and 3M sodium acetate in washing step. ● Added equal volume of isopropanol ● Used double washing (96 % and 75 % ethanol)	● Incubation of mixture on water-bath for one hour at 65°C ● Centrifuge the mixture and solutions at 17,949 × g. ● Incubation at -20°C for overnight and next day follows the steps of Doyle & Doyle, 1987 protocol.
DBP	● Two buffers (lysis buffer without CTAB and extraction buffer with CTAB) were used instead of single buffer. ● Used 0.15 gm of leaves instead of 0.5 gm. ● Increased the volume of extraction and washing buffer (1000 µL instead of 998 µL ● Increased the concentration (3 %) of CTAB and PVP-40 instead of 2 %. ● Increased the concentration of β-ME (used 0.5 % instead of 0.2 %) and volume of sodium acetate (used 5µL instead of 2µL).	● Incubation for half hour at 4°C ● Incubation on water-bath for one hour at 65°C ● Centrifuge the mixtures and solutions at 20,817 × g.

Table 3
List of Universal ISSR markers used for PCR amplification of DNA extracted from *P. crenulata*

S. No.	Primer Code	Base Sequence (5'-3')	Temperature (Tm)
1.	UBC-807	AGAGAGAGAGAGAGAGT	57.8°C
2.	UBC-826	ACACACACACACACACC	55.0°C
3.	UBC-873	GACAGACAGACAGACA	59.6°C
4.	UBC-888	[1]BDBCACACACACACACA	59.6°C
5.	UBC-890	[2]VHVGTGTGTGTGTGT	59.6°C

[1] B=Guanine/Thymine/Cytosine; D=Guanine/Adenine/Thymine
[2] H=Adenine/Cytosine/Thymine; V= Guanine/Cytosine/Adenine

was again washed with 70% ethanol (500 µL) and then centrifuged at 20,817 × g at 4°C for 5 min. The supernatant was discarded and the pellet was dried in a vacuum drier (Concentrator – 5301, Eppendorf, Hamburg, Germany). The pellet was re-suspended in high salt TE-buffer (100 µL) and left at room temperature for dissolving and then stored at -20°C for further use.

Double Buffer Protocol

Sterilized 0.15 gm leaf material was ground to a fine powder in liquid nitrogen and transferred to a 2-mL micro-centrifuge tube containing pre-chilled (4°C) EB (1 mL Lysis Buffer + 5 µL β-mercaptoethanol) and incubated for 30 min at 4°C. The tubes were then centrifuged at 4°C and 1,699 × g for 6 min and the top aqueous layer was discarded. Pre-warmed EB (1 mL Extraction Buffer + 5 µL β- mercaptoethanol) was added to the tube and incubated in a water bath at 60°C for 1 hour with constant mixing. The tubes were kept at room temperature and 500 µL of Chloroform: Iso-amyl alcohol (24:1) was added and gently inverted until a homogeneous mixture was formed. Then the tubes were centrifuged at 4°C and 20,817 × g for 7 min. The supernatant was transferred to a 1.5-mL tube, and the pellet was discarded. An equal volume (1:1) of chilled isopropanol (-20°C) was added to the supernatant and the tubes were gently inverted until DNA filaments were visible. The tubes were incubated overnight at -20°C. Next day, the tubes were centrifuged at 20,817 × g for 15 min at 4°C, and the supernatant was discarded. The pellet was washed with 96% ethanol (995 µL) and 3 M sodium acetate (5 µL) by placing the tubes on gel rocker for 45 min. Tubes were centrifuged at 20,817 × g for 5 min at 4°C and the supernatant was discarded. The pellet was rewashed with 70% ethanol (500 µL) and then centrifuged at 20,817 × g for 5 min at 4°C. Supernatant was discarded and the pellet was dried in a vacuum concentrator (AG 22331 Eppendorf, Hamburg, Germany). The pellet was re-suspended in high salt TE-buffer (100 µL) and left at room temperature for dissolving and then stored at -20°C for further use.

Qualitative and Quantitative Analysis

The quality of extracted genomic DNA from all the protocols was analyzed on 0.8 % (w/v) agarose gel using 1X Tris Boric acid EDTA (TBE) buffer and the quantity was checked at A_{260}/A_{280} nm by using electrophoresis (C.B.S. Scientific) and bio-photometer (Eppendorf, Hamburg, Germany), respectively. The agarose gel was stained by Ethidium bromide (0.5 µg/mL) and visualized under gel-documentation system (GelDoc-It Imaging system, UVP model LMS-20E, Upland, USA).

Optimization of ISSR PCR Amplification

The genomic DNA from all three protocols was checked for PCR amplification with ISSR markers using a gradient master cycler (Nexus Gradient, Eppendorf, Hamburg, Germany) with primers UBC-807, UBC-826, UBC-873, UBC-888 and UBC-890 (Table-3). The PCR was performed in a 25 µL reaction volume containing 1X *Taq* buffer, 1.25 mM $MgCl_2$, 0.15 mM each dNTPs, 0.4 µM primer, and 1 U *Taq* DNA polymerase with 20 ng/µL DNA template; the final volume was made up with 18 µL sterile nuclease-free distilled water. PCR amplification was performed with the following cycling parameters: initial denaturation at 94°C for 5 min; 40 cycles at 94°C for 30 sec, specific annealing temperature for 30 sec and 72°C for 1 min followed by a final extension at 72°C for 10 min. The temperature range for the gradient PCR was taken from 55°C to 60°C (55.0°C, 55.1°C, 55.4°C, 55.9°C, 56.6°C, 57.2°C, 57.8°C, 58.4°C, 59.1°C, 59.6°C, 59.9°C, and 60.0°C). The PCR amplification products were separated on 1.5 % (w/v) agarose gel stained with ethidium bromide (10 mg/ mL) using 1X tris-borate-EDTA (TBE) as running buffer. The separated bands were visualized under UV in Gel-Documentation System (GelDoc-It Imaging system, UVP model LMS-20E, Upland, USA).

Results

Conventional CTAB and modified activated charcoal methods did not yield promising results for *P. crenulata* and produced a sticky pellet due to contamination of polysaccharides and polyphenols. Several difficulties were encountered during the DNA isolation procedure. During the separation step, the supernatant was very viscous, difficult to be pipetted, and barely mixed with isopropanol. The pellet in CTAB method was white but was very sticky and viscous, while brown and sticky pellet was obtained in modified activated charcoal method (Figure 1). The smeared and sheared bands were observed in agarose gel and the samples remained stuck in the wells (Figure 2a (CCP & ACP)). The A_{260}/A_{280} ratio and the yield ranged from 1.34 to 2.42 and from 27.4 to 477.4 ng/ µL respectively, were also very poor. The yield and purity of extracted DNA were good in double buffer protocol, ranging from 368 to 410 ng/ µL and 1.8 to 1.84, respectively (Table 4). In this method, an intact g-DNA band, no RNA and protein contamination, no viscosity and stickiness in pellets were observed (Figure 2a (DBP)). Conventional CTAB and modified activated charcoal protocols showed no amplification (gel image is not attached), whereas a clear banding pattern with ISSR markers was observed when PCR was performed with DNA extracted from double buffer protocol (Figure 2b). The viscous and sticky pellet obtained with the conventional CTAB and activated charcoal protocols indicate the presence of impurities that interfere with the *Taq* polymerase enzyme and thus no amplification was observed. However, the additional buffer used in DBP removed the impurities at the initial step and helped in successful PCR amplification. The summary of the results are graphically represented which showing a clear visualization of the DNA concentration and purity of all employed protocols (Figure 3).

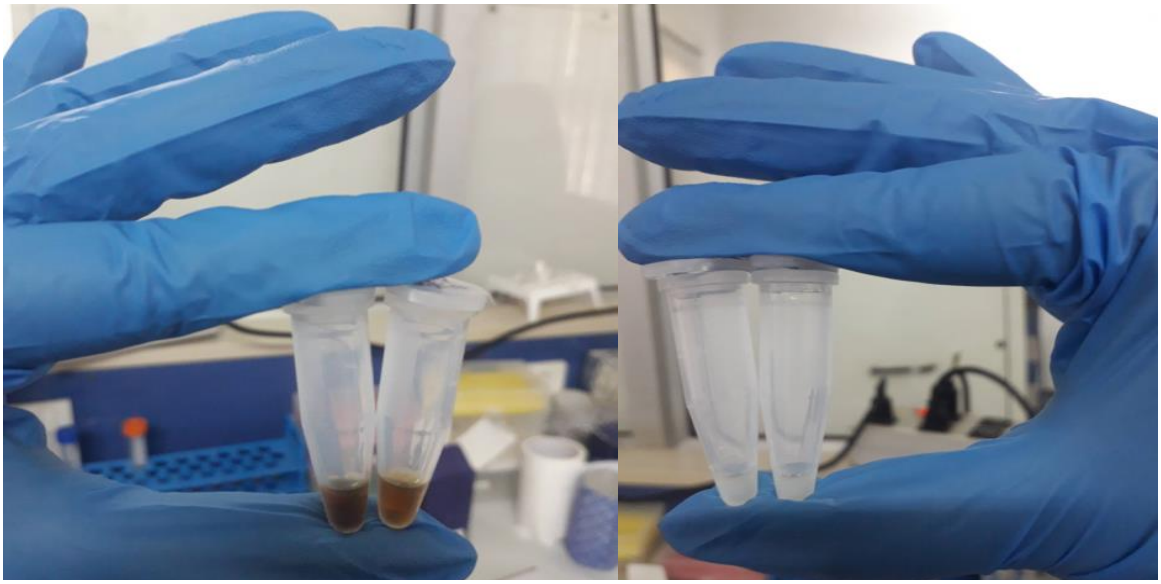


Figure 1
Comparative evaluation of DNA extraction protocols: (a) DNA extracted by ACP protocol exhibits brownish hue, while (b) Double buffer protocol yields clear, non-sticky and transparent high-quality DNA

Table 4
Comparison of concentration and absorbance ratio of DNA samples extracted from *P. crenulata* leaves using different protocols

Protocol Used	Sample Amount (gm)	Concentration (ng/ µL)	Absorbance	
			A260/A280	A260
Conventional CTAB Protocol	0.5	27.4	1.49	0.068
		39.6	2.42	-0.021
Modified Activated Charcoal Protocol	0.5	477.4	1.34	0.434
		138.2	2.23	0.099
Double Buffer Protocol	0.15	502.7	1.80	0.282
		518.0	1.82	0.550

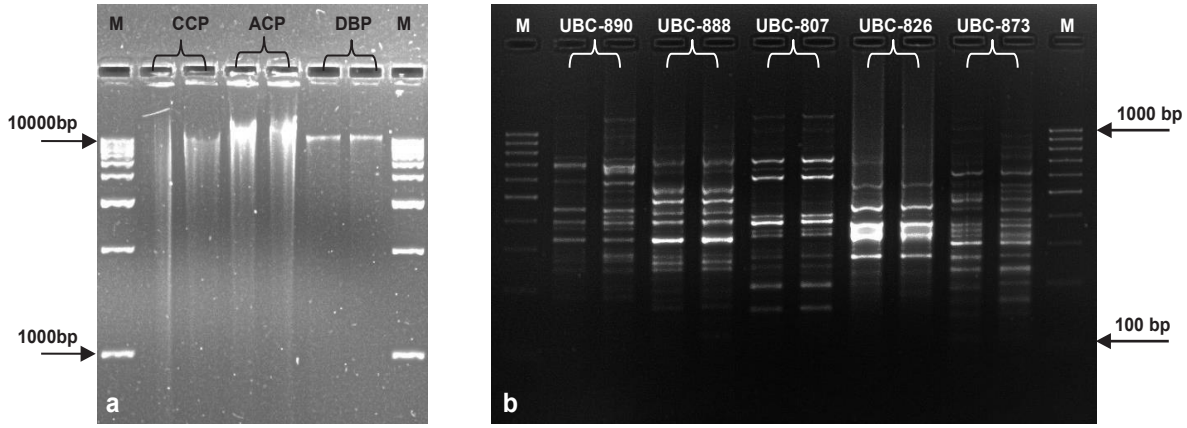


Figure 2
(a) Genomic DNA extracted from *P. crenulata* using different protocols (CCP, ACP and DBP); (b) PCR amplification in *P. crenulata* genotypes using 5 ISSR primers

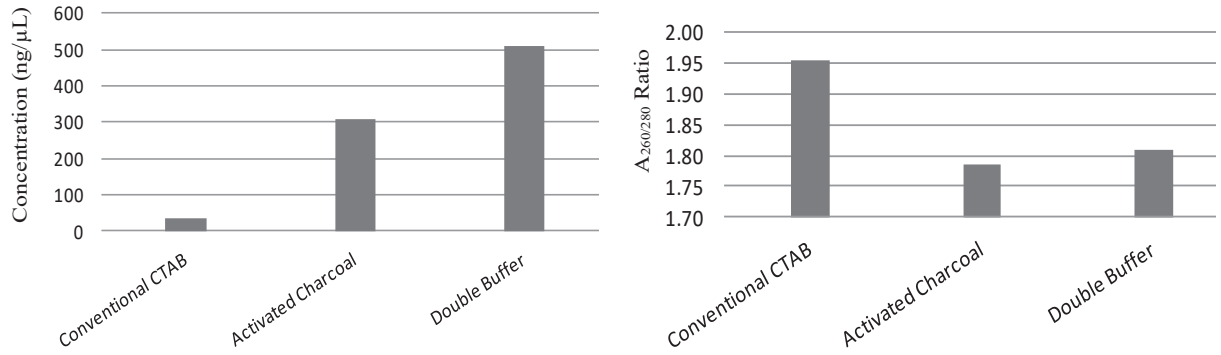


Figure 3
Summary of DNA quantitative analysis (a) DNA concentration (ng/µL) obtained with the three protocols; (b) $A_{260/280}$ ratio indicating purity of the DNA obtained from the three protocols

Discussion

The basic principle of DNA isolation is disruption of the cell wall, cell membrane, and nuclear membrane to release the highly intact DNA into solution followed by precipitation of DNA and removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols, and other secondary metabolites by enzymatic or chemical methods (Sika et al., 2015). We used three protocols for obtaining good quality of DNA from the leaves of *P. crenulata*. In conventional CTAB and modified activated charcoal protocols, sticky and brown pellets were obtained. The highly viscous, sticky and brownish pellets are challenging to handle and indicate phenolic contamination which interferes in PCR reactions by inhibiting the enzymatic activity of the *Taq* polymerase (Moreira and Oliveira, 2011). The quality and quantity of DNA recovered was also very poor in conventional CTAB and modified activated charcoal methods. The DNA extracted from these methods was not suitable for molecular marker based studies. In conventional CTAB protocol, the mechanical force exerted during grinding with liquid nitrogen disrupts the complex polysaccharide cell wall and when this cell lysate is incubated with CTAB buffer, the cell membrane is disrupted by the CTAB resulting in the release of nucleus. Plant tissue, which is rich in complex polysaccharides and secondary metabolites, interferes and co-precipitates with DNA, making the pellet sticky and viscous.

Activated charcoal is a form of carbon that has been extensively used to remove various contaminants from water, soil and air with its adsorption capacity which has proven useful in pharmaceuticals, industry and microbiology. It has also been successfully used in plant tissue culture to prevent growth inhibition from excess polyphenols released into the medium and to remove PCR inhibitors and increase the sensitivity of real-time PCR reactions. In activated charcoal protocol, we utilised the adsorption property of activated charcoal along with PVP in the extraction buffer to effectively remove polyphenols and other complex secondary metabolites from the DNA preparations isolated from *P. crenulata*. The addition of activated charcoal significantly improved the yield of DNA but could not remove the impurities to an extent which could allow PCR amplification.

Finally, the double buffer protocol was used for the genomic DNA extraction. Of all the protocols, the double buffer protocol provided best results in terms of yield as well as purity. In this protocol, an additional buffer (lysis buffer without CTAB) was used prior to CTAB buffer. The lysis buffer helped in the removal of polysaccharides and polyphenols from the cell lysate before disruption of cell membrane, thus preventing the co-precipitation of these contaminants with the DNA. The polysaccharides from the cell wall are removed along with the supernatant prior to the addition of CTAB buffer. For the first two protocols, 0.5 gm of leaf sample was collected, while for the double buffer, the sample amount was just 0.15 gm. The amount of leaf sample was reduced to 0.15 gm in order to decrease viscosity of the sample. Since, the leaves of *P. crenulata* contain high phenolic compounds, therefore, by reducing the sample amount the viscosity was observed negligible. The Lysis Buffer (without CTAB) helped in breaking the cell wall and removing polyphenols from the crude DNA extracts. Since, PVP helps in the removal of phenolic compounds (Porebski et al., 1997; Khanuja et al., 1999), therefore, its concentration was increased from 2 % to 3 % in the double buffer protocol, which removed the phenolic compounds from the DNA extracts and prevented the discoloration of DNA pellet due to oxidation of polyphenols. It has also been widely studied (Khanuja et al., 1999; Deng et al., 2019) that the high amount of β-mercaptoethanol is successful in removing the polyphenols by breaking the disulfide bonds of the proteins present in the plant DNA extracts, therefore the amount of β-mercaptoethanol was increased from 0.2 % to 0.5 % in the modified protocol (DBP). The reduced amount of leaf tissue along with increased amount of PVP and β-mercaptoethanol in LB helped to reduce viscosity of the crude extracts by removing the impurities along with the supernatant after half-hour incubation at 4°C. EB (with CTAB) helped to disrupt cell membranes. CTAB along with PVP reduced the impact of secondary metabolites and

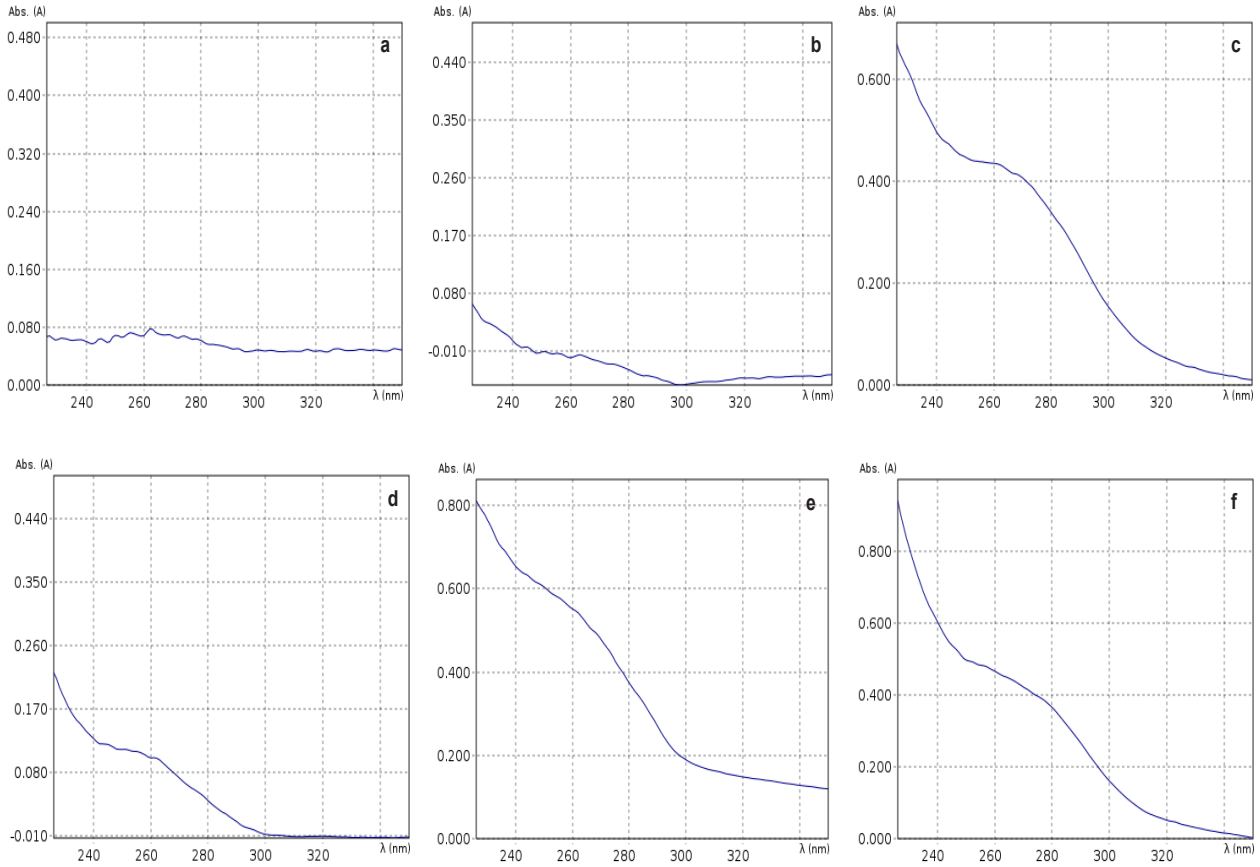


Figure 4
Graphical representation of absorbance (A260) pattern in *P. crenulata* DNA samples extracted using different protocols (a) & (b): Conventional CTAB protocol; (c) & (d): Modified activated charcoal protocol; and (e) & (f): Double buffer protocol

polysaccharides in the process. 3 % CTAB and 3 % PVP with 0.5 % β-ME helped to remove all residual phenols, polysaccharides, and secondary metabolites from the DNA extracts. The yield and purity of the extracted DNA by double buffer protocol were very good with yield ranging from 368 to 410 ng/µL and $A_{260/280}$ ratio ranging between 1.8 and 1.84, respectively and the values at A_{260} also supported the results (Figure 4). The value of 1.8 indicates a high purity of the extracted DNA while values below and above 1.8 denotes contaminations of proteins and RNAs, respectively (Rawat et al., 2013).

It is studied (Weishing et al., 1995; Ivanova et al., 2008) that sheared DNA can directly or indirectly interfere with the enzymatic reactions such as ligation and restriction digestion and create hindrance in PCR amplification and RNA contamination can be problematic in sequencing reactions. The pellets obtained in the modified protocol (DBP) were white and non-sticky, the DNA bands were intact and no smearing was observed in the gel indicating no RNA contamination which validated the high purity of extracted DNA. Moreover, the samples did not stick in the wells and the problem of high viscosity was also resolved. Due to the contamination and sheared DNA obtained from conventional CTAB and modified activated charcoal protocol, no amplification was observed during PCR optimization whereas a clear banding pattern was observed when PCR was performed with DNA extracted from double buffer protocol. To check the efficacy of the extracted genomic DNA from DBP, PCR amplification was carried out. The primer gave the intense and sharp bands which validated the standardized protocol. The results indicated that the double buffer protocol successfully removed the polysaccharides, polyphenols and secondary metabolites from the samples and yielded high quality genomic DNA which was suitable for DNA-based molecular marker studies. The proposed approach may also be useful for extraction of DNA from mature leaf samples which contain high levels of polyphenols, secondary metabolites, and polysaccharides, since in some species, collecting young leaves is quite challenging for DNA extraction. The adopted methodology is described in the form of flow chart (Figure 5).

Conclusion

For carrying out the genetic studies, DNA extraction is an essential part. DNA extraction is not easy in plants having rich phenolic compounds, polysaccharides and secondary

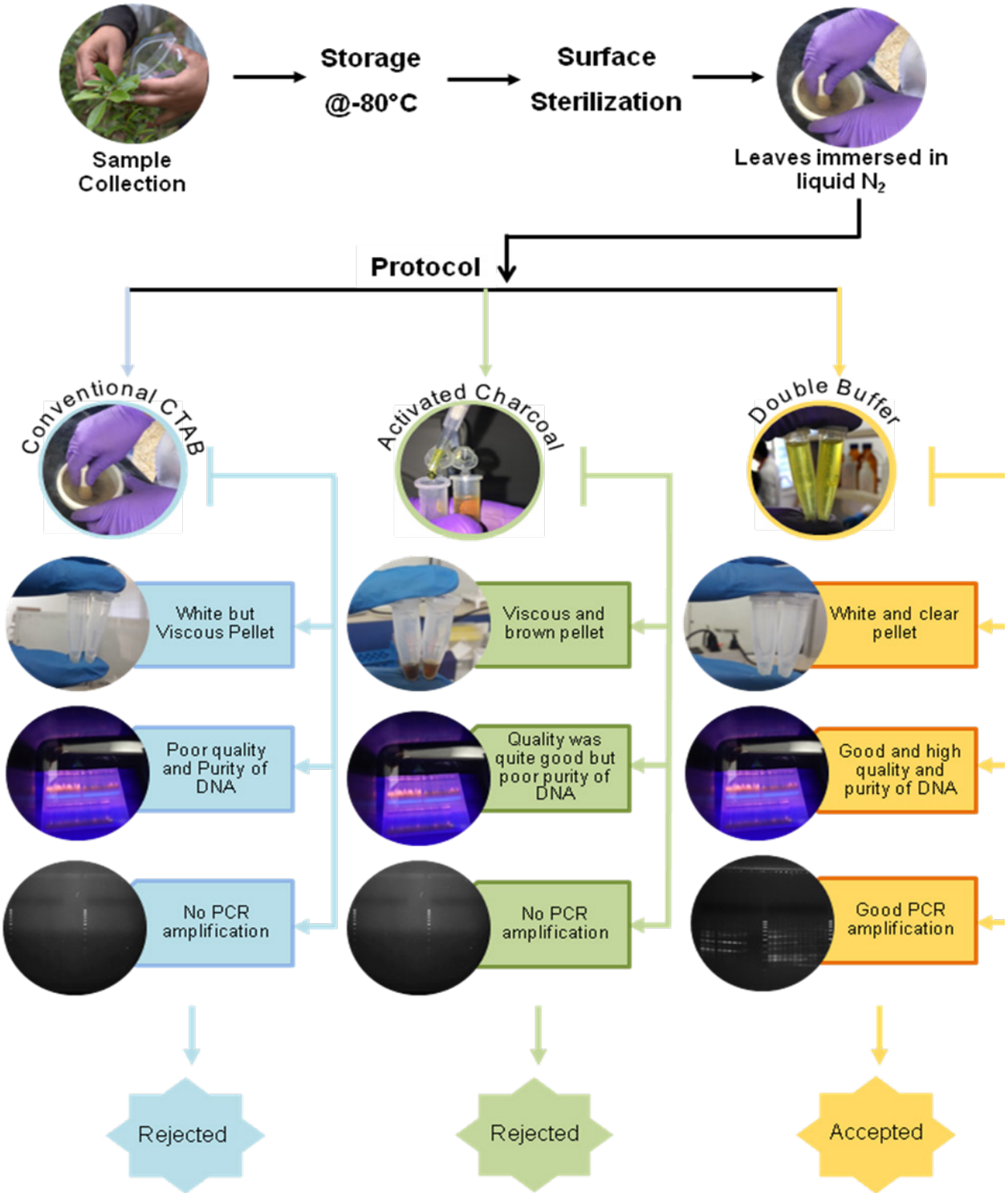


Figure 5
Flow chart of the methodology adopted in the study

metabolites and *P. crenulata* is one of them. The results indicated that of all the three protocols, double buffer protocol yielded higher and good DNA concentration and was successful in removing polyphenols, polysaccharides, and secondary metabolites from plant tissues, whereas the protocols using CTAB and modified activated charcoal were not satisfactory. The rapid, simple, efficient, and reliable DNA extraction method developed in the present study can also be used in other species of the Rosaceae family having high polysaccharides and polyphenols. The modified protocol yielded large amounts of pure genomic DNA from small amount of tissue in *P. crenulata* and was found to be suitable for PCR amplification. Therefore, this method is recommended for various molecular analytical procedures such as next generation sequencing analysis. The DNA isolation from young leaves is easy in mostly protocols but this protocol is effective for both young as well as mature leaves yielding high quality genomic DNA. The DBP protocol might be suitable for matured and dried leaves, if young leaf tissue is not available. This method is suitable for various medicinal plants which are generally rich in polyphenols and secondary metabolites. There are no genetic studies in *P. crenulata* so far and the developed protocol will certainly help in wide array of molecular and phylogenetic analysis, genome sequencing and genetic engineering of this complex species. Due to its multipurpose uses, this species has over-exploited by the locals and industrialist. Thus, it is crucial to consider this species' genetic sight in order to conserve it. The present study yields a valuable insight which will aid in understanding its population genetic structure and would help in devising conservation strategies for the species in future. It might be useful in other species of the family Rosaceae and also in other families' species.

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Conflict of Interest

The article has been read by each author and there is no conflict of interest.

Data Availability

This manuscript did not generate any genetic resource in the form of nucleic acid or protein sequences, SNPs, expression data, etc. which could be deposited in the databases. The ISSR sequences used here for the DNA fingerprinting work are already in the public domain. Further, all the data generated herein are presented in the manuscript.

Author Contributions

Haripriya Kavidayal – Conceptualization, sample collection, data curation, writing an original draft, methodology.
Anita Rawat – Manuscript writing, methodology, supervision.
Shanti Saroj – Sample collection, material preparation, methodology.
Harish Singh Ginwal – Manuscript writing, validation, supervision

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