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Original Research Article

Effects of Temperature and Centrifugation Conditions on DNA Isolation From Different Leaf Ages In *Aconitum heterophyllum* Wall. ex Royle

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Abstract

Aconitum heterophyllum Wall. ex Royle, commonly known as Atees, is a critically endangered medicinal herb endemic to the Northwestern Himalayan region, valued for its therapeutic alkaloid atisine. The extraction of high-quality genomic DNA from this species presents significant challenges due to elevated concentrations of polysaccharides, polyphenolic compounds and secondary metabolites inherent to high-altitude medicinal plants. This study systematically evaluated the effects of leaf developmental stage (young, middle-aged and mature) and centrifugation conditions on DNA yield and purity using a modified CTAB protocol. Samples were collected from Ghesh village, Chamoli district, Uttarakhand and DNA was extracted under varying parameters: 10,000 rpm at 25°C, 12,000 rpm at 30°C and 15,000 rpm at 35°C. Spectrophotometric and electrophoretic analyses revealed that young leaves produced the highest DNA yield (847 µg/mL) and optimal purity ratios (A/A = 1.82, A/A = 2.15) under 10,000 rpm at 25°C, whereas higher centrifugation speeds and temperatures resulted in reduced quality. Optimal DNA extraction was achieved at 10,000 rpm and 25°C, demonstrating that elevated centrifugation speeds and temperatures inversely correlate with DNA quality. The inclusion of 1% PVP and 0.2% -mercaptoethanol in the CTAB buffer effectively mitigated interference from secondary metabolites. RAPD-PCR amplification using primer OPA-1 (CAGGCCCTTC) generated clear and distinct banding patterns (7-9 bands) from optimally extracted DNA, confirming its suitability for molecular applications. These findings establish a reproducible, cost-effective protocol for high-quality genomic DNA extraction from *A. heterophyllum*, providing methodological guidance for molecular characterization, genetic diversity assessment and conservation strategies for endangered Himalayan medicinal flora.

Keywords: *Aconitum heterophyllum, CTAB extraction, centrifugation optimisation, leaf age variation, DNA purity, RAPD markers, medicinal plants, Himalayan biodiversity, conservation genetic.*

1. Introduction

The Aconitum genus (Ranunculaceae) comprises approximately 250 species worldwide, 30 documented in India. *Aconitum heterophyllum* Wall. ex Royle, commonly known as Atees or Ativisha, is a critically endangered medicinal herb native to the Northwestern Himalayan region, thriving at elevations between 2,800-4,500 m asl (Singh *et al*., 2008; Nautiyal *et al*., 2009; Bhagyawant, 2016; Negi *et al*., 2021; Purohit *et al*. 2021). Unlike its toxic congeners, this species contains non-toxic diterpene alkaloids, particularly atisine (0.4%), making it valuable in traditional medicine for treating gastrointestinal disorders, fever and as an immunomodulatory agent (Ukani *et al*., 1996; Tai *et al*., 2015; Regmi *et al*., 2020). With market prices exceeding 7,500 per kilogram, the species faces severe population decline due to unsustainable harvesting and habitat degradation (Murray Thompson 1980; Nayar Sastry 1987; IUCN, 1993; Chauhan *et al*. 2023; Sharma *et al*., 2021). Molecular characterisation using DNA-based markers has become essential for conservation genetics, genetic diversity assessment and authentication of medicinal plants (Gopalakrishnan *et al*., 2014; Zhao *et al*., 2015). However, DNA extraction from *A. heterophyllum* presents significant challenges due to high concentrations of polyphenolic compounds, polysaccharides and secondary metabolites that interfere with DNA isolation, causing oxidation, co-precipitation and enzymatic inhibition (Williams *et al*. 1990; Khanuja *et al*., 1999; Srivastava *et al*., 2010; Thakre *et al*., 2021). Modified CTAB protocols incorporating polyvinylpyrrolidone (PVP) and -mercaptoethanol have shown promise for recalcitrant medicinal plants (Kumar *et al*., 2018), yet systematic optimisation for *A. heterophyllum* remains undocumented (Cole and Kuchenreuther 2001; Faisal *et al*., 2012; Hao *et al*., 2013) Leaf developmental stage significantly influences DNA quality, with young tissues generally yielding superior results due to lower secondary metabolite accumulation and reduced lignification (Nagori *et al*., 2014; Deore *et al*., 2014). Additionally, centrifugation parameters and temperature critically affect DNA integrity, with high speeds potentially causing shearing and elevated temperatures accelerating degradation (Puchooa, 2004; Mengliang *et al*., 2007; Mitka *et al*., 2007; Hatwal *et al*., 2011). Despite their importance, the interactive effects of leaf age, centrifugation speed and temperature on DNA extraction efficiency from *A. heterophyllum* have not been systematically investigated. This study evaluated DNA isolation from three leaf developmental stages (young, middle-aged and mature) under varying centrifugation conditions (10,000, 12,000 and 15,000 rpm at 25°C, 30°C and 35°C respectively) using a modified CTAB protocol. DNA quality was assessed through spectrophotometry, gel electrophoresis and RAPD-PCR amplification using primer OPA-1. The findings establish evidence-based protocols for molecular characterization of this endangered Himalayan medicinal plant, supporting conservation genetics and breeding programs.

2. MATERIALS AND METHODS

2.1 Study Site and Sample Collection

Fresh leaf samples of *Aconitum heterophyllum* were collected in July 2022 from cultivated fields in Ghesh village (30°07'47.3196"N; 79°43'23.664"E; 3,200 m asl), Chamoli district, Uttarakhand, India. Leaves were categorised into three developmental stages based on morphological characteristics and canopy position: (i) Young leaves : fully expanded, bright green, tender leaves from upper canopy with no chlorosis; (ii) Middle-aged leaves : moderately mature, dark green leaves from mid-canopy; and (iii) mature leaves : older, darker leaves from lower canopy showing age-related changes. Samples were immediately frozen in liquid nitrogen and stored at -20°C until DNA extraction. The experimental work was conducted at the Biochemistry and Molecular Biology Laboratory, High Altitude Plant Physiology Research Centre (HAPPRC), Hemvati Nandan Bahuguna Garhwal Central University, Srinagar (Garhwal), Uttarakhand, India.

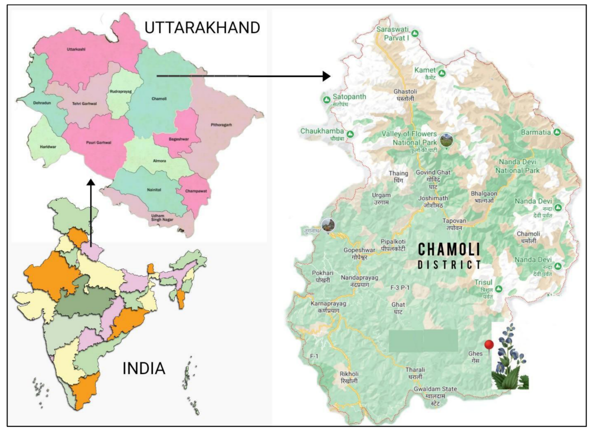

Figure 1. Location of sample collected, Ghesh village of Chamoli district, Uttarakhand, India

2.2 Chemicals and Equipment

All chemicals were molecular biology grade, procured from HiMedia Laboratories (Mumbai), Sigma-Aldrich and Merck Life Sciences. Key reagents included CTAB, PVP, EDTA, Tris-HCl, NaCl, -mercaptoethanol, chloroform, isoamyl alcohol, isopropanol, ethanol, RNAse A and agarose. Major equipment included refrigerated centrifuge (REMI 612 LAG), thermal cycler (Bio-Rad T100™), UV-VIS spectrophotometer (Systronics AU-2701), gel electrophoresis system (LA1075 Hi-Gel Run

100) and gel documentation system (Bio-Rad Universal Hood II).

2.3 DNA Extraction Buffer Preparation

Standard stock solutions (0.5 M EDTA pH 8.0, 1 M Tris-HCl pH 8.0, 5 M NaCl) were prepared using autoclaved double-distilled water following standard protocols. The modified DNA extraction buffer (2× CTAB) was prepared fresh with the composition detailed in Table 1. PVP (1%) and -mercaptoethanol (0.2%) were added immediately before use to prevent oxidative degradation.

2.4 DNA Extraction Protocol

DNA extraction followed a modified CTAB method (Doyle Doyle, 1987) optimized for *A. heterophyllum*. Fresh leaf tissue (200 mg) from each developmental stage was ground to fine powder in liquid nitrogen using pre-chilled mortar and pestle and immediately transferred to 2 ml microcentrifuge tubes. Pre-warmed extraction buffer (2 ml, 65°C) was added to each tube and mixed thoroughly. Samples were incubated at 65°C for 45 minutes with gentle inversion every 5-10 minutes to facilitate cell lysis and membrane solubilization. After cooling to room temperature, equal volume (2 ml) of chloroform and isoamyl alcohol (24:1, CIA) was added and mixed by gentle inversion. Samples were centrifuged under three different parameter sets: (A) 10,000 rpm at 25°C for 10 minutes; (B) 12,000 rpm at 30°C for 10 minutes; (C) 15,000 rpm at 35°C for 10 minutes. The upper aqueous phase (1.5-1.8 ml) was carefully transferred to fresh tubes using wide-bore tips. RNAse A (5 µL of 10 mg/mL) was added and samples incubated at 37°C for 40 minutes. A second CIA extraction was performed using identical centrifugation parameters and the aqueous phase was transferred to fresh tubes. One volume of chilled isopropanol (-20°C) was added, mixed gently and the samples incubated overnight at -20°C for DNA precipitation. Following centrifugation at respective treatment parameters for 10 minutes at 4°C, supernatant was discarded, and pellets were washed twice with 1 ml chilled 70% ethanol. After brief centrifugation (5 minutes), ethanol was removed and pellets were air-dried at room temperature for 20-30 minutes. Dried DNA pellets were resuspended in 300 µL TE buffer (pH 8.0) by gentle pipetting, incubated at 37°C for 30 minutes and stored at -20°C.

2.5 DNA Quality Assessment

2.5.1 Agarose Gel Electrophoresis

DNA integrity was assessed by 0.8% agarose gel electrophoresis in 1× TAE buffer containing ethidium bromide (5 µL/100 ml gel). DNA samples (3-4 µl) mixed with 6× loading dye were loaded alongside a 100 bp DNA ladder (HiMedia). Electrophoresis was conducted

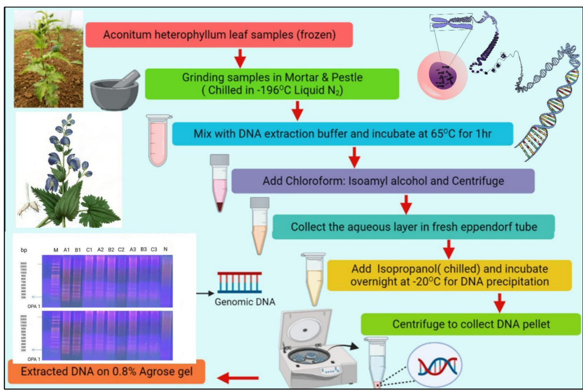

Figure 2. Process of DNA Extraction as per protocol

at 80 V for 90-120 minutes. Gels were visualised under UV transillumination (302 nm) and documented using the Bio-Rad gel documentation system. DNA quality was evaluated based on band integrity, intensity and absence of smearing.

2.5.2 Spectrophotometric Analysis

DNA samples were diluted 1:100 in TE buffer and absorbance measured at 260 nm, 280 nm and 230 nm using UV-VIS spectrophotometer with TE buffer as blank. DNA concentration was calculated as: DNA concentration (µg/ml) = A × dilution factor × 50. Purity was assessed using A/A ratio (protein contamination indicator; optimal: 1.75-1.85) and A/A ratio (polysaccharide contamination indicator; optimal: 2.0-2.2).

2.6 RAPD-PCR Amplification

PCR amplification was performed using RAPD primer OPA-1 (5'-CAGGCCCTTC-3'). Each 20 µL reaction contained components as detailed in Table 3. Amplification was conducted in Bio-Rad T100™ thermal cycler using cycling conditions in Table 4.

PCR products (6 µL with 2 µL loading dye) were separated on 2.0% agarose gels in 1× TAE buffer with ethidium bromide. Electrophoresis was conducted at 80 V for 2 hours with 100 bp ladder for size determination. Gels were visualized under UV light and documented. Negative controls (no template DNA) were included to detect contamination.

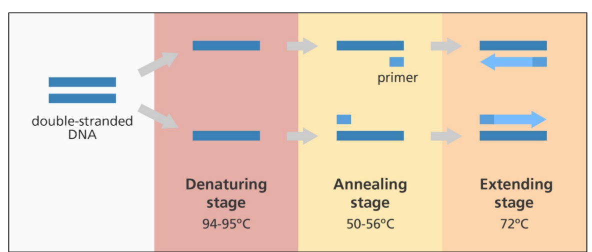

Figure 3. Three steps of PCR cycle

Table 1. Composition of DNA Extraction Buffer (100 mL)

Component	Stock Concentration	Volume Added	Final Concentration
CTAB	10%	20 ml	2%
Tris-HCl (pH 8.0)	1 M	10 ml	100 mM
EDTA (pH 8.0)	0.5 M	4 ml	20 mM
NaCl	5 M	28 ml	1.4 M
PVP	Powder	1 g	1% (w/v)
-mercaptoethanol	Pure	200 µl	0.2% (v/v)
Distilled water	-	To 100 ml	-

Note: PVP and -mercaptoethanol added fresh before use

Table 2. DNA Extraction Variables and Experimental Design

Leaf Stage	Treatment Code	Centrifugation Speed	Temperature	Replicates
Young	A1	10,000 rpm	25°C	3
Young	A2	12,000 rpm	30°C	3
Young	A3	15,000 rpm	35°C	3
Middle-aged	B1	10,000 rpm	25°C	3
Middle-aged	B2	12,000 rpm	30°C	3
Middle-aged	B3	15,000 rpm	35°C	3
Mature	C1	10,000 rpm	25°C	3
Mature	C2	12,000 rpm	30°C	3
Mature	C3	15,000 rpm	35°C	3

2.7 Data Analysis

DNA extraction quality was categorized based on gel electrophoresis and spectrophotometric purity as: Best (sharp bands, A/A = 1.75-1.85, A/A = 2.0-2.2), Good (distinct bands, ratios slightly outside optimal), Moderate (visible bands with smearing), Poor (faint bands) and Very Poor (barely visible bands). Quantitative data including DNA concentration and purity ratios, were expressed as mean ± standard deviation from three biological replicates. Comparative analysis across treatments identified optimal extraction parameters.

3. RESULTS

3.1 DNA Quality Assessment and Yield Analysis

DNA extraction from *Aconitum heterophyllum* leaves exhibited significant variation based on leaf developmental stage and centrifugation parameters. Young leaves consistently produced whitish to cream-colored DNA pellets that resuspended readily in TE buffer, while mature leaves yielded dark brown, compact pellets resistant to complete resuspension, indicating substantial polyphenolic contamination. Agarose gel electrophoresis revealed distinct quality patterns across treatments (Figure 1). Young leaves extracted at 10,000 rpm and 25°C (sample A1) exhibited sharp, intense high molecular weight bands with minimal smearing, indicating intact genomic DNA. Middle-aged leaves under identical conditions (B1) showed comparable but slightly reduced intensity, while mature leaves (C1) displayed moderate quality with visible low molecular weight smearing. At elevated parameters (15,000 rpm, 35°C), DNA quality deteriorated substantially across all leaf ages, with mature leaves (C3) showing barely detectable DNA and extensive degradation products. Spectrophotometric analysis provided quantitative validation of these observations (Table 5). Young leaves at optimal conditions (10,000 rpm, 25°C) yielded the highest DNA concentration (847 ± 45 µg/mL) with superior purity ratios (A/A = 1.82 ± 0.03; A/A = 2.15 ± 0.08), indicating minimal protein and polysaccharide contamination. DNA yield and purity progressively declined with increasing centrifugation speed and temperature across all leaf ages. Young leaves maintained acceptable quality even under suboptimal conditions, while mature leaves failed to achieve satisfactory purity ratios under any tested parameters, with the poorest performance at 15,000 rpm and 35°C (287 ± 48 µg/mL; A/A = 1.52 ± 0.09). Direct comparison revealed that young leaves outperformed middle-aged leaves by 10.7% and mature leaves by 48.1% in DNA yield under optimal conditions. This superiority became more pronounced under harsh extraction parameters, with young leaves yielding 55.6% more DNA than mature leaves at 15,000 rpm and 35°C. The A/A ratio, particularly sensitive to polysaccharide contamination, showed a dramatic decline in mature leaves, dropping from 1.91 to 1.59 between treatments C1 and C3.

3.2 RAPD-PCR Amplification Validation

PCR amplification using RAPD primer OPA-1 successfully generated distinct banding patterns, validating DNA suitability for molecular applications. Amplification products ranged from approximately 300 to 1,500

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Table 3. PCR Reaction Components

Component	Volume per Reaction	Final Concentration
10× PCR buffer (with MgCl)	2.0 µL	1×
dNTP mix (10 mM each)	0.4 µL	200 µM
Primer OPA-1 (10 µM)	1.0 µL	0.5 µM
Taq DNA polymerase (5 U/µL)	0.2 µL	1.0 U
Template DNA (50 ng/µL)	2.0 µL	∼100 ng
Nuclease-free water	14.4 µL	-
Total volume	**20.0 µL**	-

Table 4. PCR Thermal Cycling Conditions

Step	Temperature	Duration	Cycles
Initial denaturation	94°C	4 minutes	1
Denaturation	94°C	30 seconds	-
Annealing	36°C	45 seconds	40
Extension	72°C	90 seconds	-
Final extension	72°C	7 minutes	1
Hold	4°C		-

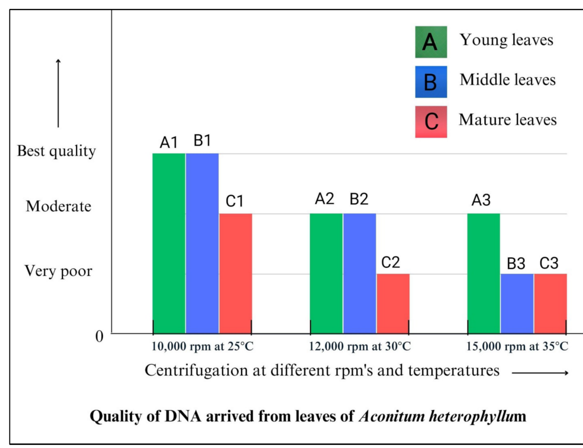

Figure 4. Graphical representation of DNA Quality arrived from different leaf ages of *Aconitum heterophyllum*

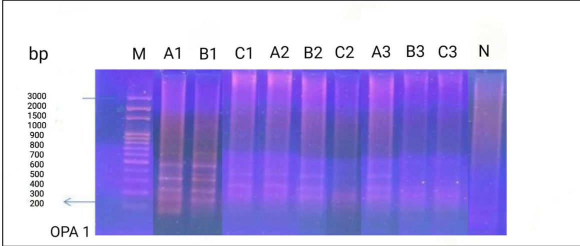

Figure 5. DNA bands using OPA from leaf samples of *Aconitum heterophyllum*

bp, with band intensity and number correlating directly with DNA quality assessments. Sample A1 produced 7-9 clear, intense bands, indicating robust amplification from high-quality template DNA. Middle-aged leaf DNA under identical conditions (B1) yielded 6-8 bands with slightly reduced intensity, while mature leaf DNA (C1) generated 4-6 bands with noticeably diminished intensity and background smearing. At elevated centrifugation parameters, amplification efficiency declined progressively. Young leaf DNA at 15,000 rpm and 35°C (A3) produced 4-5 moderate-intensity bands, while mature leaf DNA under identical conditions (C3) showed minimal amplification with only 1-2 barely detectable bands and extensive background smearing. Negative controls showed no amplification, confirming the absence of reagent contamination. The consistent correlation between spectrophotometric purity, gel assessment and PCR success validated the reliability of quality metrics employed.

4. DISCUSSION

4.1 Interactive Effects of Leaf Age and Centrifugation Parameters

This investigation demonstrates that leaf developmental stage and centrifugation conditions critically determine DNA extraction success in *Aconitum heterophyllum*. Young leaves achieved 48% higher yield and superior purity compared to mature leaves under optimal conditions, consistent with lower secondary metabolite accumulation and reduced lignification in actively growing tissues (Nagori *et al*., 2014; Bhagyawant, S. S. 2016; Kumar *et al*., 2018). The age-dependent quality decline reflects biochemical challenges inherent to mature tissues. High polyphenolic content in older leaves undergoes oxidation during tissue disruption, producing reactive quinones that irreversibly bind DNA, causing strand breaks and polymerase inhibition (Loomis, 1974; Bisset, N. G., 1981; Khanuja *et al*., 1999). Elevated polysaccharide levels further compromise quality through co-precipitation, forming viscous complexes resistant to purification. Optimal parameters (10,000 rpm at 25°C) yielded 20-49% higher DNA concentrations than harsh conditions (15,000 rpm at 35°C), challenging assumptions that higher centrifugal forces improve recovery. High-speed centrifugation

Table 5. DNA Yield and Purity from Different Leaf Ages Under Varying Centrifugation Conditions

Sample Code	Leaf Stage	Centrifugation Parameters	DNA Concentration (µg/ml)	A/A	A/A	Quality Rating
A1	Young	10,000 rpm, 25°C	847 ± 45	1.82 ± 0.03	2.15 ± 0.08	Best
A2	Young	12,000 rpm, 30°C	723 ± 38	1.78 ± 0.04	1.95 ± 0.11	Good
A3	Young	15,000 rpm, 35°C	612 ± 52	1.71 ± 0.06	1.82 ± 0.09	Moderate
B1	Middle-aged	10,000 rpm, 25°C	765 ± 41	1.79 ± 0.04	2.08 ± 0.10	Good
B2	Middle-aged	12,000 rpm, 30°C	598 ± 47	1.73 ± 0.05	1.88 ± 0.12	Moderate
B3	Middle-aged	15,000 rpm, 35°C	438 ± 59	1.64 ± 0.07	1.71 ± 0.14	Poor
C1	Mature	10,000 rpm, 25°C	572 ± 55	1.69 ± 0.06	1.91 ± 0.13	Moderate
C2	Mature	12,000 rpm, 30°C	394 ± 61	1.58 ± 0.08	1.68 ± 0.15	Poor
C3	Mature	15,000 rpm, 35°C	287 ± 48	1.52 ± 0.09	1.59 ± 0.18	Very Poor

Values represent mean ± standard deviation from three biological replicate

Table 6. Summary of DNA Quality Assessment Across Experimental Treatments

Centrifugation Parameters	Young Leaves	Middle-aged Leaves	Mature Leaves
10,000 rpm at 25°C	Best quality: High yield, optimal purity, strong PCR amplification (7-9 bands)	Good quality: Moderate yield, acceptable purity, good amplification (6-8 bands)	Moderate quality: Lower yield, borderline purity, moderate amplification (4-6 bands)
12,000 rpm at 30°C	Good quality: Moderate yield, good purity, good amplification (6-8 bands)	Moderate quality: Reduced yield, acceptable purity, moderate amplification (4-6 bands)	Poor quality: Low yield, poor purity, weak amplification (3-4 bands)
15,000 rpm at 35°C	Moderate quality: Reduced yield, acceptable purity, moderate amplification (4-5 bands)	Poor quality: Low yield, poor purity, weak amplification (2-3 bands)	Very poor quality: Very low yield, very poor purity, minimal amplification (1-2 bands)

generates mechanical shearing forces fragmenting genomic DNA, while elevated temperatures accelerate DNase activity and chemical degradation. Room temperature centrifugation minimizes thermal stress while maintaining adequate phase separation, optimizing extraction efficiency and DNA integrity preservation.

4.2 Protocol Optimisation and Molecular Validation

The modified CTAB protocol incorporating 1% PVP, 0.2% -mercaptoethanol and 1.4 M NaCl effectively mitigated contamination challenges. PVP sequesters polyphenols through hydrogen bonding, -mercaptoethanol maintains reducing conditions inhibiting quinone formation, and high salt concentration enhances polysaccharide solubility (Fang *et al.*, 1992; Sharma Via, 2002). Reducing -mercaptoethanol from 2% to 0.2% improved purity without compromising yield, minimizing residual contamination affecting downstream applications. Successful RAPD-PCR amplification validated DNA suitability for molecular applications. Sample A1 produced 7-9 clear bands, confirming utility for genetic diversity assessment, marker-assisted selection and DNA barcoding essential for conservation programs. The correlation between spectrophotometric purity and PCR success establishes protocol reliability for *A. heterophyllum* molecular characterization.

4.3 Comparative Context and Conservation Implications

These findings align with studies in other high-altitude medicinal plants. Srivastava *et al*. (2010) and Negi *et al*., (2022) reported similar challenges with *A. heterophyllum*, emphasizing young leaf superiority and PVP effectiveness. Comparable results in Eclipta alba and Aloe barbadensis validate this approach for recalcitrant medicinal species (Kumar *et al*., 2018; Deore *et al*., 2014). Beyond methodology, this protocol addresses critical conservation needs. *A. heterophyllum* faces severe population decline from unsustainable harvesting, with market prices exceeding 7,500/kg driving overexploitation (Sharma *et al*., 2021). Reliable DNA extraction enables: (i) genetic diversity assessment for conservation prioritization; (ii) DNA barcode development for authentication; (iii) marker-assisted breeding for sustainable cultivation; and (iv) germplasm banking for ex situ conservation. The optimized protocol: young leaves, 10,000 rpm at 25°C, modified CTAB buffer - provides reproducible, cost-effective DNA suitable for molecular applications while remaining accessible for resource-limited biodiversity hotspot laboratories. This evidence-based methodology establishes a technical foundation for conservation genetics, sustainable cultivation programs and biodiversity preservation strategies essential for endangered Himalayan medicinal flora.

5. Conclusion

This investigation establishes evidence-based protocols for genomic DNA extraction from *Aconitum heterophyllum*, addressing critical methodological gaps in the molecular characterisation of high-altitude medicinal plants. Young leaves yielded superior DNA quality (847 µg/mL, A/A = 1.82) compared to middle-aged and mature tissues under optimal conditions (10,000 rpm at 25°C). Elevated centrifugation speeds and temperatures inversely correlated with DNA quality, with mature leaves showing 49.8% yield reduction at 15,000 rpm and 35°C. RAPD-PCR validation confirmed DNA suitability for molecular applications, with young leaf samples generating 7-9 distinct amplification bands. The modified CTAB protocol incorporating 1% PVP, 0.2% -mercaptoethanol and optimized centrifugation parameters effectively mitigated polyphenol and polysaccharide contamination challenges inherent to *A. heterophyllum*. These findings provide a methodological foundation for conservation genetics, genetic diversity assessment, DNA barcoding and breeding programs supporting sustainable cultivation and germplasm preservation of this critically endangered Himalayan medicinal herb. The protocol's applicability extends to other high-altitude medicinal plants with similar phytochemical challenges, advancing molecular research capabilities for endangered alpine flora.

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