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Method

Comparative study of DNA and RNA extraction methods for high-quality nucleic acid isolation from *Cullenia exarillata* A. Robyns

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Abstract

Isolation of high-quality DNA and RNA from plants with high polysaccharide and secondary metabolite content is typically difficult, particularly in the case of trees. Metabolites commonly undergo co-precipitation with RNA and DNA, resulting in degradation of their quality. *Cullenia exarillata* leaf samples were subjected to various DNA and RNA extraction techniques, and the resulting data were compared and analysed. The isolation of high-quality DNA and RNA is crucial for the advancement of molecular and biotechnological techniques that aim to preserve the endemic status of *C. exarillata* and this research is to establish an efficient procedure for extracting DNA and RNA from *C. exarillata*. This method will make molecular and genomic research easier in forestry and conservation fields. In this research, we evaluated various methods for extracting high quality DNA and total RNA from *C. exarillata* tree, incorporating minor modifications in standard procedure. To acquire DNA and RNA of superior quality, a comparison was made between conventional DNA and RNA extraction methods and a variety of commercial kits that revealed the conventional technique yielded DNA samples of superior purity and concentration. It was discovered that combining modified commercial and conventional procedures yielded RNA with exceptionally high concentration and purity. The Agilent 2100 Bioanalyzer and NanoDrop spectrophotometer ensure the impeccable purity of the nucleic acids generated via these procedures. Additionally, the application of agarose gel electrophoresis unveiled unique bands. Further investigation was conducted to validate the purity and amplification of the DNA and RNA that were collected. This study clarifies a method for extracting sufficient and high-quality amounts of DNA and RNA from *C. exarillata*; future research on this plant will greatly benefit from knowing this information.

Keywords: CTAB, Comparative study, *Cullenia exarillata*, DNA, RNA extraction, High quality DNA, High quality RNA.

1. Introduction

DNA and RNA isolation are commonly used techniques in molecular biology laboratories. The process used to perform the extraction of DNA and RNA is dependent upon the specific characteristics of the plant material. The published work of Doyle and Doyle is widely used in molecular genetics laboratories worldwide as standard operating procedure [1]. Depending on the particular plant or plant material, this method may need to be modified or require more procedures. Achieving consistent and satisfactory results across all plant species using the same process is not always possible, emphasizing the importance of a standardized protocol to provide smooth and reliable research outcomes. Designing a standardized protocol specifically for *C. exarillata* is crucial to overcoming the unique challenges posed by its physical and chemical characteristics.

C. exarillata is an endemic canopy tree found in the evergreen forests of the Western Ghats. During its blooming phase, the tree becomes a hub of activity and a crucial resource for several species of arboreal mammals, including the endangered lion-tailed macaque [2][3][4]. Many mammals consume the seeds of *C. exarillata*, both on the ground and in the canopy. Consequently, discovering new seedlings proves to be immensely challenging. Due to the uniform age structure of the population, the development of new plants is limited [5]. The flowering seasons of *C. exarillata* are eagerly awaited by the lion-tailed macaques, and certain sources indicate the necessity of *C. exarillata's* flowering in the forest for the presence of these macaques. The blossoming flowers throughout the forest create a captivating spectacle. Moreover, some research suggests a mutualistic relationship between the tree and the lion-tailed macaque. The tree also serves as a food source for various animal species, proving crucial from August to November during regional food crises [6][7]. Therefore, safeguarding the long-term survival of the tree is crucial, and delving into the genetic diversity of *Cullenia* populations will aid in understanding gene flow and implementing preventive measures against inbreeding depression, while also facilitating gene flow in highly endemic and threatened animals. Consequently, the pro-

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tocols for DNA and RNA isolation have to be optimized to suit plant species from different genera, rendering them suitable for further diversity studies [8].

Reports indicate the presence of secondary metabolites in leaves such as polysaccharides and polyphenols [9], which act as contaminants, causing interference in DNA and RNA isolation [10]. These contaminants can lead to poor DNA yield, and it will affect the PCR amplification. While the height of the tree presents logistical challenges, the primary issue is the interference caused by secondary metabolites during DNA extraction. Variations in plant secondary metabolites, such as polysaccharides, polyphenols, and other substances, across different plant species, have been observed, posing challenges in isolating high-quality DNA and RNA from plants [11][12][13]. Additionally, the presence of mucilage in plant samples complicates DNA and RNA extraction, resulting in low yields and inadequate quality of genetic material [14][15]. Notably, the effectiveness of DNA and RNA isolation methods varies among different plant species. While several techniques exist for isolating DNA and RNA from plant tissues, they often yield insignificant amounts or genetic material of inconsistent quality. Many of these extraction methods are adaptations of cetyltrimethyl ammonium bromide (CTAB) extraction, which exhibit limitations across different crops and exhibit variations in time and cost. The variability in the CTAB protocol stems from differences in cell wall structure, mitochondria, cellulose, and the presence of secondary metabolites. Although commercial DNA extraction kits are available, they often yield minimal genetic material and are prohibitively expensive, especially for developing countries. For challenging plant species, column-based extraction kits have been effective in obtaining contaminant-free, high-quality DNA and RNA, albeit with residual genetic material left on the column. DNA and RNA isolation kits do not work efficiently for all plant samples, particularly those rich in secondary metabolites. The buffers provided with these kits are highly specific, limiting the flexibility for protocol modifications. Additionally, silica-based columns may not completely remove DNA during RNA isolation or vice versa, leading to cross-contamination. Consequently, DNase or RNase treatment is often required to ensure purity. To enhance DNA and RNA yield and simplify the purification process, updates were made to the DNeasy Plant Mini Kit [16][17]. This study aimed to compare the quality and quantity of DNA and RNA obtained from various extraction methods, striving to develop a faster, simpler, and reliable DNA and RNA extraction method that meets the requirements of PCR based genetic analysis and transcriptome sequencing.

2. Materials and Methods

2.1. Plant Material

Leaf samples from *C. exarillata* were collected randomly in Ponmudi, Thiruvananthapuram, Kerala, India (Geographical Coordinates: Lat: 8°75.99'N; Lon: 77.11°69'E) and preserved at -80°C for subsequent DNA extraction. Simultaneously, *Cullenia* fruit seeds were collected and subjected to germination to acquire fresh, young leaf samples for RNA isolation. The plant was cultivated in the conservatory located at the Jawaharlal Nehru Tropical Botanic Garden and Research Institute in Palode, Thiruvananthapuram, Kerala, India. The seeds took almost 02 Months for germination. Once the plants develop beyond the five-leaf stage, tender leaves are collected for RNA isolation.

2.2. DNA extraction

Origin - Method: *C. exarillata* genomic DNA was extracted using the commercially available Origin Plant Genomic DNA Kit following the manufacturer's instructions, taking approximately 1.5–2 hours. The resulting product was divided into aliquots and stored at -20°C.

Favorgen - Method*: C. exarillata* genomic DNA extraction was performed utilizing the FavorPrep Plant Genomic DNA Extraction Mini Kit according to the manufacturer's protocol, requiring approximately 2–3 hours. The final product was divided into aliquots and stored at -20°C.

Qiagen - Method: Genomic DNA from *C. exarillata* was isolated using the DNeasy Plant Mini kit from Qiagen, following the manufacturer's guidelines. This method, based on Qiagen's silica gel membrane technology employing the 'bind-wash-elute' technique, took approximately 1.5 to 2.5 hours and final product were stored at -20°C.

CTAB-Based Method: The CTAB protocol, adapted from Murray et al. [1980] [18], Approximately 150 mg of plant leaf tissue is ground in liquid nitrogen to form a fine powder, which is subsequently transferred into a 2 mL autoclaved Eppendorf tube containing 1 mL of prewarmed (10 to 15 minutes) CTAB buffer with 2% β-mercaptoethanol and a pinch of PVP (Polyvinylpyrrolidone). PVP can be incorporated either into the CTAB buffer solution or during the grinding of the leaf tissue. The mixture is supplemented with 4 µL of Proteinase K, gently inverted for 1 minute, and incubated at 65°C for 20 minutes, with mixing at 5-minute intervals to ensure thorough homogenization. Following incubation, the sample is centrifuged at 13,000 rpm for 5 minutes, and the supernatant is transferred to a fresh 2 mL Eppendorf tube. To facilitate the removal of contaminating organic material, an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) is added to the supernatant, which is then gently mixed by inversion and centrifuged at 10,000 rpm for 15 minutes. The aqueous supernatant is carefully transferred to a new tube, and a second extraction step is performed by adding an equal volume of Chloroform: Isoamyl alcohol (24:1), followed by inversion mixing (20–25 times) to form an emulsion. The sample is then centrifuged at 13,000 rpm for 10 minutes. The supernatant is decanted, and DNA is precipitated by adding an equal volume of pre-chilled (-20°C) Isopropanol and one volume of Sodium acetate. The mixture is incubated at -20°C overnight or at -80°C for 2 hours to allow DNA precipitation.

After precipitation, the sample is centrifuged at 13,000 rpm for 15 minutes to pellet the DNA. The supernatant is discarded, and the DNA pellet is washed with 100 µL of pre-chilled 100% ethanol at 10,000 rpm for 1 minute to remove residual organic solvents. Two additional washes with 70% ethanol are performed, each at 10,000 rpm for 1 minute. The ethanol is carefully decanted, and the DNA pellet is air-dried at room temperature until it becomes transparent. The dried pellet is resuspended in 100 µL of double-autoclaved Millipore water, followed by the addition of 1 µL of RNase. The mixture is incubated at 37°C for 30 minutes or at 65°C for 10–15 minutes to degrade any residual RNA. The final DNA solution is stored at -20°C or -80°C for long-term preservation. This proto-

col provides a reliable method for extracting high-quality genomic DNA from plant tissues, suitable for downstream applications such as PCR, sequencing, and cloning.

2.3. RNA extraction

CTAB using the QIAzol Combined RNeasy Plant Mini Kit: The CTAB protocol utilizing the QIAzol Combined RNeasy Plant Mini Kit, adapted from Nadiya et al. [2014] [19], was employed for the extraction of total RNA from young, tender *C. exarillata* leaves. Prior to commencing the extraction method, all glassware, spatulas, pipettes, tubes, gel boxes, and solutions were treated to be RNase-free using DEPC water. 100 mg of leaf tissue was ground into a fine powder in a mortar and pestle with liquid nitrogen. Pre-warmed RNA extraction buffer (1 mL + 10 µL of β-mercaptoethanol) in 2 mL tubes was prepared at 65°C before adding plant tissues. The powdered tissue was combined with the extraction buffer using a spatula chilled in liquid nitrogen, followed by vortexing to suspend it in the extraction buffer. Subsequently, the tubes were tightly closed and placed in a 65°C water bath, with intermittent vortexing for at least 25 min. After 2 min of cooling at room temperature (RT), 640 µL of chloroform: isoamyl alcohol (24:1) was added to the tubes, vortexed for 2 min, and then centrifuged for 15 min at 13,000 rpm at 4°C. The top aqueous layer was collected using a new tube. To the aqueous supernatant, 720 µL of 8M lithium chloride (LiCl) was added, mixed thoroughly, and stored at -80°C for an hour. Following centrifugation (HermLe Z216 MK, USA) for 30 min at 16,000 rpm at 4°C, the supernatant was removed, and an RNeasy column (Qiagen, Germany) was utilized to proceed with the extraction process. Additionally, 700 µL of QIAzol Lysis Reagent was added to the tube containing the pellet and pipetted to dissolve the pellet. The homogenate was left at RT (15–25°C) for 5 min, followed by the addition of 140 µL of chloroform and shaking for 15 sec. After centrifugation for 15 min at 12,000 rpm at 4°C, the top aqueous layer (650 µL) was transferred to a fresh collection tube. This was followed by the addition of 1.5 volumes (about 975 µL) of 100% ethanol and subsequent mixing by pipetting. The sample, including any precipitate, was loaded into an RNeasy Mini Spin column (Qiagen, Germany) and centrifuged at 8000 x g for 15 sec at RT. The flow-through was discarded, and the previous step was repeated with the remaining sample. DNase digestion was carried out on the column by adding 350 µL of buffer RW1 and centrifuging for 15 sec at 10,000 rpm. Subsequently, 80 µL of DNase I incubation mix was added onto the membrane and incubated at 20 to 30°C for 15 min. After centrifugation for 15 sec at 8000 x g, the flow-through was discarded. Washing steps were performed by adding and centrifuging 500 µL of buffer RW1 and buffer RPE successively. To elute the RNA, 15 µL of RNase-free water was added onto the RNeasy Mini spin column membrane and centrifuged for 1 min at 10,000 rpm. This elution step was repeated with a sec volume of 15 µL RNase-free water. Finally, the resulting RNA was aliquoted and stored at -80°C for further use.

Combined CTAB and RNeasy Plant Mini Kit Method: Total RNA will be isolated from different cell lines using the combined CTAB and miRNeasy Mini Kit method. Prior to initiating the extraction procedure, it is essential to ensure all glassware, spatulas, pipettes, tubes, gel boxes, and solutions are RNase-free using DEPC water. 100 mg of leaf tissue will be ground into a fine powder using a mortar and pestle with liquid nitrogen. RNA extraction buffer (1 mL plus 10 µL of β-mercaptoethanol) in 2 mL tubes will be pre-warmed to 65°C before adding the plant tissues. The ground substance will be transferred to a 2 mL tube previously cooled in liquid nitrogen, followed by the addition of the pre-chilled extraction buffer. After vortexing to suspend the contents, the tube will be incubated at 65°C, with samples being vortexed six to eight times during the incubation period. Following a 2 min cooling period at room temperature, samples will be centrifuged at 10,000 rpm for 2 min. The supernatant will then be transferred to a fresh tube. Subsequently, an equal volume of freshly prepared chloroform-isoamyl alcohol will be added to the supernatant, followed by centrifugation at 4°C for 15 min at 12,000 rpm. The upper aqueous layer will be transferred to a new tube, mixed with an equivalent volume of isopropanol or absolute ethanol, and incubated at -80°C for two hours. Subsequently, 700 µL of the sample, including any precipitate, will be loaded into a RNeasy Mini Spin column. Centrifugation will be carried out at 10,000 rpm for 1 min at room temperature, discarding the flow-through. The DNase digestion will then proceed on the column by washing with 350 µL of buffer RWT (made with ethanol) and centrifuging for 1 min at 10,000 rpm, discarding the flow-through. The column will be dried by further centrifugation in a new collection tube. Following this, 70 µL of DNase will be added directly onto the RNeasy Mini centrifuge column membrane and left at 20 to 30°C for 15 min. Subsequent centrifugation with 350 µL of buffer RW1 and 700 µL of RPE will be carried out at 10,000 rpm for 1 min each, discarding the flow-through after each step. The column will be transferred to a new 2 mL tube and centrifuged for 2 min at 10,000 rpm to dry the membrane. Finally, 30 µL of RNase-free water will be added directly onto the RNeasy Mini spin column membrane, centrifuged at 10,000 rpm for 1 min to elute the RNA, and the eluted RNA collected into the same tube. This elution step will be repeated with a sec volume of 20 µL RNase-free water. The resulting RNA will be aliquoted and stored at -80°C.

2.4. Spectrophotometric analyses of DNA and RNA

The quality and concentration of the isolated DNA and RNA were assessed spectrophotometrically using an N50 Nanophotometer (IMPLEN, USA) at wavelengths of 260 nm and 280 nm. The absorbance ratios at A260/280 (typically ~ 1.84 DNA and ~2.0 for RNA) and A260/230 (ideally between 2.0 and 2.2) were utilized to evaluate the quality of DNA and RNA samples.

2.5. Agarose gel electrophoresis

Purity and integrity assessment of total DNA and RNA bands were conducted through electrophoresis on 0.8% and 1% agarose gels stained with ethidium bromide. Electrophoresis was performed at 70 V using a tank buffer of 1X TAE. After 30 min, gel analysis was carried out using Geli software on the UVP EC 3 Chemi HR 410 Imaging System (UVP, California).

2.6. Polymerase Chain Reaction (PCR) for DNA analysis

In the polymerase chain reaction, 40 nanograms of *Cullenia* genomic DNA were employed. Table 1 contains the

Table 1. List of primers used in this study.

Name	Primer Sequence	Annealing Tm
SCOT 28	5'-CAACAATGGCTACCACCA-3'	50°C
SCOT 32	5'-CCATGGCTACCACCGCAC-3'	50°C
SCOT 22	5'-AACCATGGCTACCACCAC-3'	50°C
SCOT 27	5'-ACCATGGCTACCACCGTG-3'	50°C
SCOT 21	5'-CCATGGCTACCACCGGCC-3'	50°C
SCOT 41	5'-CAACAATGGCTACCAGCA-3'	50°C

Table 2. Summary of DNA extraction methods used in this study.

DNA extraction method	Basis and format	Maximum DNA yield (ng DNA/mg sample)	DNA purity A260 nm/A280 nm ratio	DNA purity A260 nm/A230 nm ratio
Origin	Silica membrane binding, Spin column format	5.25ng	0.97	0.97
Favorgen	Silica membrane binding; spin-column format	22.25ng	1.40	0.52
Qiagen	Silica membrane binding; spin-column format	42.35ng	1.90	1.90
CTAB	Solution-based; selective precipitation of DNA	341.40ng	1.80	2.30

list of primers used in the PCR amplification for this study. The total volume of the PCR reaction was 15 µL. Each reaction tube contained 7.5 µL EmeraldAmp GT PCR Master Mix, 15 picomoles SCOT primer, 40 ng/µL DNA and the remaining sterile water. PCR amplification was conducted in a thermal cycler (Veriti™ 96-Well Thermal Cycler, Applied Biosystems, California) under the following parameters: Reactions initiated with a 5-min denaturation at 94°C, followed by 35 cycles of 1-min denaturation at 94°C, 1-min annealing at 50°C, 2-min extension at 72°C, and a final extension step of 10 min at 72°C. The amplified products were separated in 1.5% agarose gels containing ethidium bromide, in 1X Tris-borate EDTA buffer, and analyzed using a gel documentation system (UVP, UK). A 100bp DNA ladder (OrionX) was loaded alongside the samples to facilitate comparison of resultant band sizes.

2.7. Assessment of RNA RIN value

Three measurements aimed at evaluating RNA integrity and quality: denaturing formaldehyde agarose gel electrophoresis inspection, determination of the ribosomal RNA subunit ratio (26S/18S), and quantification of the RNA Integrity Number (RIN) along with its equivalent values (RINeq) [18,19]. The RIN was measured using the plant RNA Nanochip assay in the Agilent 2100 Bioanalyzer with the plant RNA configuration option in the Bioanalyzer program release B.02.07. Additionally, the RIN was assessed using the Agilent 2200 TapeStation's RNA ScreenTape assay following the manufacturer's guidelines [20] .

2.8. Statistical analysis

Statistical analyses were performed using single leaf sample with four replicates using SPSS software (version 22). The differences in DNA concentrations among four DNA extraction protocols were analyzed using the General Linear Model (GLM) Univariate ANOVA. DNA extraction protocol was used as a fixed factor, and biological samples were added as a random factor to control for variability. Significant differences between protocols were further analyzed using Tukey's post hoc multiple-comparison test. A p-value less than 0.05 was considered statistically significant.

A multivariate analysis of variance (MANOVA) was performed with single leaf sample with four replicates to evaluate whether RNA extraction protocol methods (CTAB using the QIAzol Combined MiRNeasy Mini Kit vs. Combined CTAB and miRNeasy Mini Kit method) had a significant effect on RNA concentration and RNA Integrity Number (RIN). The independent variable was extraction protocol, while the dependent variables were RNA concentration (ng/µL) and RIN value. Assumptions of normality and homogeneity were assessed and satisfied.

3. Results

In this study, we compared different extraction methods to obtain high-quality DNA and RNA from leaves, given their easy accessibility. The CTAB-based method yielded higher-quality DNA than the kit-based methods and showed superior DNA quantity when measured using the Nanodrop method. The 260/280 nanometre absorbance ratios for DNA was 1.8, indicated high purity with minimal contamination from secondary metabolites. The modified CTAB method exhibited a DNA yield ranging from 120ng to 130 ng, obtained from 100 mg of homogenized leaf powder, sufficient for conducting PCR. However, the 260/280 ratio was found to range between 1.2 and 2.0. Table 2 summarizes the DNA yield and purity ranges for all methods used.

The method of extraction influenced DNA quality, with the presence of polysaccharides, lipids, polyphenols, CTAB, EDTA, and Tris potentially affecting DNA purity. Both the Qiagen and CTAB extraction methods yielded DNA with high purity ratios of 1.8 to 1.9 (260/280) and 1.9 to 2.3 (260/230), respectively. While purity levels were sufficient, concentration remains a critical parameter for successful gene amplification [26]. In this protocol, RNase is introduced at the final step, following DNA resuspen-

sion, to optimize RNA removal. During the initial lysis step with CTAB, various salts and detergents present in the buffer can interfere with RNase activity, rendering it less effective. Additionally, plant-derived compounds such as phenolics and polysaccharides may bind to RNase, further inhibiting its function. If RNase is added during the precipitation stage, it could be removed during the chloroform extraction, reducing its effectiveness. By resuspending the DNA pellet in water before the RNase treatment, any interfering salts, detergents, and plant compounds are removed, allowing RNase to work more effectively. Furthermore, by this stage, the DNA is already purified, making RNA more accessible for degradation. Therefore, it is generally more effective to add RNase after DNA resuspension, ensuring efficient RNA removal and resulting in high-quality DNA suitable for downstream applications.

The Qiagen method provided higher purity with a lower concentration, whereas the DNA concentration was higher in samples extracted using the CTAB method. Gel electrophoresis of the extracted DNA samples revealed distinct bands corresponding to their respective molecular weights (Fig. 1). A 260/280 ratio falling between 1.93 to 2.27 indicates moderate contamination levels [25]. Ratios lower than or equal to 1.6 may suggest the presence of proteins, phenol, or other contaminants that significantly absorb around 280 nm. Additionally, the 260/230 ratio, predicted to be between 2.0 and 2.2 for "pure" DNA, ranged between 2.00 and 2.30 in this study. In this present study, DNA isolation using the conventional DNA extraction method produced higher quality DNA when compared to commercial kits. Although commercial kits are faster and more convenient, they may not be suitable for all tissue types and are often more expensive, depending on the brand and kit size. In contrast, the conventional method not only yielded purer DNA but also allowed for protocol modification based on the characteristic of the tissue. Additionally conventional methods are more cost effective, making them a practical choice for laboratories with limited resources. Despite requiring more time and effort, the conventional methods provided reliable and high-quality results. These findings highlight that conventional methods remain effective and, in some cases, more suitable than commercial kits.

Regarding RNA extraction, we compared two methods for isolating high-quality RNA from *C. exarillata.* CTAB-based kit methods yielded high quantity RNA (119.5 ng, 183.9 ng) (Table 3). The resulting RNA showed distinct 25S and 18S rRNA bands with brightness and no noticeable smearing due to degradation, indicating RNA integrity using the CTAB-based kits (Fig. 2). Commonly used A260/A280 and A260/230 ratios of 2.0 to 2.2 indicate high RNA purity [27]. Results from the CTAB-based kit methods demonstrated A260/A280 ratios of 2.0–2.1 and

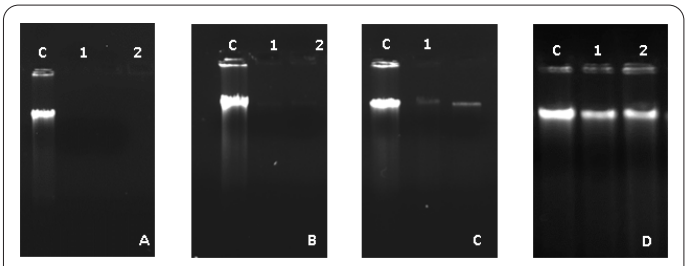

Fig. 1. Agarose gel electrophoresis showing genomic DNA preparation of two *C. exarillata* leaves (lanes 1–2) and Control (lanes C). DNA extractions using the different extraction methods (A), Favorgen (B), Origin (C), Qiagen (D), CTAB.

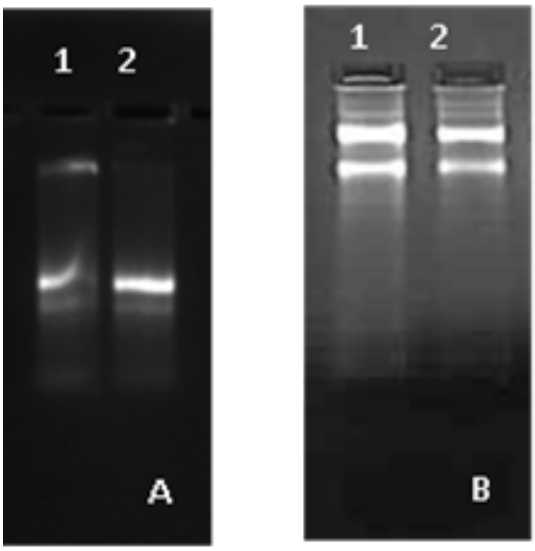

Fig. 2. Agarose gel electrophoresis showing total RNA preparation of two *C. exarillata* leaves (lanes 1–2). RNA extraction using the 2 different extraction methods (A) CTAB using the QIAzol Combined MiRNeasy Mini Kit, (B) Combined CTAB and miRNeasy Mini Kit method.

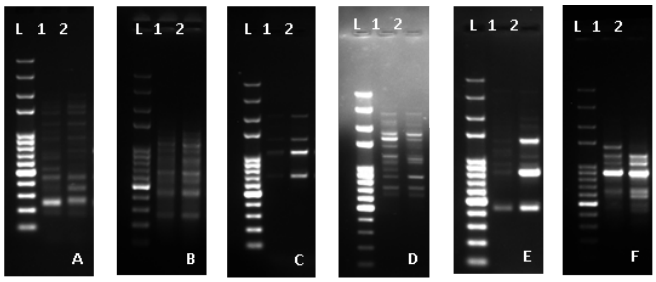

Fig. 3. Gel picture of PCR product of isolated from DNA *C. exarillata*leaf samples in the figure are indicating 100bp ladder, 1 and 2 indicating DNA samples respectively. PCR amplification using different SCOT primers (A) SCOT 22, (B) SCOT 28, (C) SCOT 41, (D) SCOT 21, (E) SCOT 27 and (F) SCOT 32.

Table 3. Summary of RNA extraction methods used in this study.

RNA extraction method	Basis and format	Maximum RNA yield (ng RNA/mg sample)	RNA purity A260 nm/A280 nm ratio	RNA purity A260 nm/A230 nm ratio	RIN Value
CTAB using the QIAzol Combined MiRNeasy Mini Kit	Solution-based; Silica membrane binding; spin-column format	119.5ng	1.9	2.4	6.7
Combined CTAB and miRNeasy Mini Kit method	Solution-based; Silica membrane binding; spin-column format	183.9ng	2.1	2.4	7.9

1.9-2.0, showing effectiveness in avoiding protein contamination. Additionally, A260/A230 ratios of 2.2 and 2.4 indicate high purity. Although no significant difference was observed in RNA concentration between the two extraction protocols.

Further analysis of RNA integrity using an Agilent 2100 Bioanalyzer revealed RIN values ≥ 7 (Table 3) for RNA from CTAB using the QIAzol Combined RNeasy Plant Mini Kit, indicating no degradation of RNA (Fig. 4 & 5). RIN values above 7.0 signify intact, high-quality RNA samples suitable for gene expression quantification by RT-qPCR or sequencing [28]. Conversely, other methods revealed RIN values below 7.

Finally, the efficiency of the extracted DNA was analyzed via PCR amplification using SCOT primers. The PCR results displayed distinct and strong bands in all samples analyzed (Fig. 3), indicating the presence of high-quality DNA extracted using the modified CTAB-based DNA extraction procedure, crucial for PCR efficiency.

Statistical analysis indicated the GLM univariate ANOVA indicated significant differences in DNA concentration among the tested DNA isolation protocols. Post hoc analyses revealed that CTAB extraction protocol yielded significantly higher DNA concentrations compared to Protocols Origin, Favorgen, and Qiagen ($p < 0.001$). Additionally, Protocol Qiagen resulted in significantly higher DNA concentrations compared to Protocol Origin ($p = 0.031$). However, no significant differences were detected between Protocols Origin and Favorgen ($p = 0.624$), or between Protocols Favorgen and Qiagen ($p = 0.233$). Protocol CTAB was thus identified as the more suitable protocol for obtaining higher DNA concentrations (Table 4).

The MANOVA showed a statistically significant overall difference in dependent variables between RNA extractions methods. Univariate ANOVA results indicated a significant effect of protocol on RIN value ($F = 18.34$, $p = 0.003$), where CTAB using the QIAzol Combined MiRNeasy Mini Kit method yielded higher RIN values. However, there was no significant difference in RNA concentration between extraction methods ($F = 2.98$, $p = 0.121$) (Table 5).

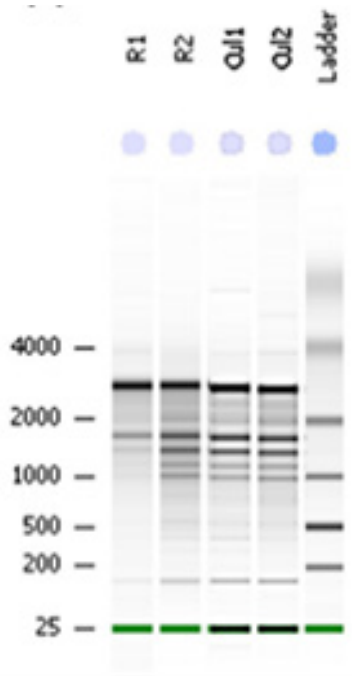

Fig. 4. Pseudo-gel image produced using an Agilent 2100 Bioanalyser, showing the results of RNA extracted from *C. exarillata* leaf samples. RNA extraction using the 2 different extraction methods (Cul1) and (Cul2) CTAB using the QIAzol Combined MiRNeasy Mini Kit, (R1) and (R2) Combined CTAB and miRNeasy Mini Kit method.

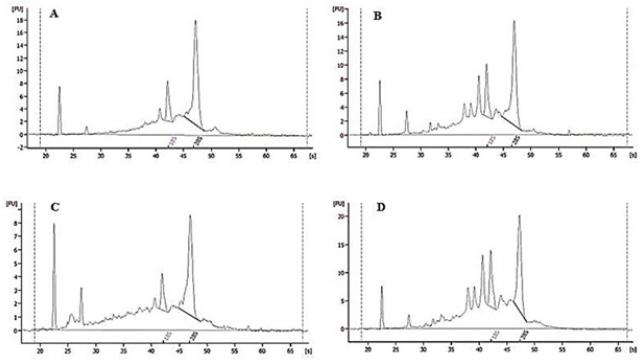

Fig. 5. Agilent 2100 Bioanalyser electropherogram graphs showing RNA extracted from the 2 different extraction methods (A) and (B) CTAB using the QIAzol Combined MiRNeasy Mini Kit, (C) and (D) Combined CTAB and miRNeasy Mini Kit method can be seen 18S RNA subunit 28S large subunit at specific area.

4. Discussion

In most molecular biology laboratories, the CTAB-based DNA and RNA isolation procedures are frequently

Table 4. Statistical comparison of DNA extraction methods.

Protocol Comparison	Mean Difference (ng/µl)	Std. Error	p-value
Origin vs Favorgen	-8.31	6.79	0.624
Origin vs Qiagen	-22.06*	6.79	0.031
Origin vs CTAB	-344.73**	6.79	<0.001
Favorgen vs Qiagen	-13.74	6.79	0.233
Favorgen vs CTAB	-336.42**	6.79	<0.001
Qiagen vs CTAB	-322.68**	6.79	<0.001

NB: Data represent mean differences in DNA concentration (ng/µl) between indicated protocols; *p < 0.05; **p < 0.001 indicate statistically significant differences (Tukey's HSD test).

Table 5. Summary of Anova results comparing RIN values and RNA concentration between RNA extraction protocols.

Dependent Variable	CTAB using the QIAzol Combined MiRNeasy Mini Kit Mean (SD)	Protocol 2 Mean (SD)	F-value	p-value
RIN Value	7.20 (0.45)	6.08 (0.37)	18.34	0.003
RNA Concentration	114.8 (3.3)	257.8 (135.5)	2.98	0.121

utilized [21]. While numerous protocols exist for isolating nucleic acids, many are species-specific and not universally applicable to all plants or tissues, necessitating a standardized procedure for extracting high-quality DNA and RNA from various sources. Molecular work often prioritizes high-quality DNA and RNA over quantity [22,23]. Although various modifications to the CTAB procedure have been proposed for extracting DNA and RNA from Malvaceae family members, these methods tend to be time-consuming and costly [24]. The QIAGEN spin-column method proved particularly effective for DNA extraction, yielding higher DNA quantities compared to other methods. [29]. There are numerous rapid DNA extraction methods are available for a variety of plant tissues. A single DNA isolation method may not be suitable for all tree species, particularly those with high polysaccharide content, such as grape (*Vitis* spp), apple (*Malus* spp), pears (*Pyrus* spp) and conifers [30][31]. The modified CTAB method is an effective and reliable technique for isolating high-quality DNA from various plant species. Unlike traditional methods, it also isolates RNA from the same tissue, making it ideal for a wide range of molecular analyses [32]. This study provides an optimized protocol to extract high-quality DNA and RNA which will aid in gene diversity studies and gene annotation. This established protocol will increase the accuracy of molecular analyses which improve the understanding of the genome of the tree species. It will help to improving the strategies for conservation management, protecting genetic resources, and aiding further studies in *C. exarillata*.

The current study effectively demonstrates a straightforward, reproducible, cost-effective, and efficient approach to obtaining uncontaminated, high-quality DNA and RNA from Cullenia leaves. The genetic material extracted can be applied to various subsequent analyses, including polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), genome sequencing, RNA-Seq, and other molecular investigations. These findings hold considerable significance for further research concerning this particular plant species.

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