



A CTAB protocol for obtaining high-quality total RNA from cinnamon (*Cinnamomum zeylanicum* Blume)

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Abstract

Cinnamomum zeylanicum Blume is an endemic Sri Lankan species commonly known as Ceylon cinnamon or true cinnamon. It is considered the king of spices in addition to its medicinal benefits. Despite recent scientific evidence on its medicinal properties and the industrial demand, cinnamon breeding and crop improvement are not been improved to the expectation. It is mainly due to the limited availability of the genomic information of cinnamon, linked with technical challenges caused by abundant secondary metabolites in all plant parts. Therefore, obtaining high-quality RNA is the fundamental step of transcriptomic analysis and the gene discovery process of cinnamon. We have optimized a CTAB based protocol for high-quality RNA extraction from different cinnamon tissues at various maturity stages collected from the field. Regular pH around 8 and the presence of Polyvinylpyrrolidone (PVP) in CTAB buffer increased the viscosity of the cinnamon lysate. Adjusting the pH of the lysis buffer to 6–6.5 reduced the viscosity of lysate while chloroform precipitates protein efficiently at the adjusted pH with no phenol. Therefore, this protocol excludes PVP and phenol extraction steps. Nanodrop spectrophotometer, gel electrophoresis, and bioanalyzer readings confirmed the quality of extracted RNA. RNA-seq libraries prepared were sequenced with Illumina Sequencing by synthesis technology and obtained good quality data to be used for transcriptomic analysis.

Keywords Ceylon cinnamon · Cinnamaldehyde · Gene expression · Phenol · Polyvinylpyrrolidone (PVP)

Introduction

Gene expression analyses have become the standard laboratory procedure to understand how plants grow and complete the life cycle, and to learn their response to biotic and abiotic stresses and environmental conditions. While the procedures are optimized for model plant species such as

Arabidopsis (Kaul et al. 2000), rice (Matsumoto et al. 2005) and tomato (Mueller et al. 2009), no standard procedures for many non-model species. *Cinnamomum zeylanicum* Blume also known as Ceylon cinnamon or true cinnamon is one such species that gained recent attention due to its health benefits and culinary value. Both leaf and bark of cinnamon are commercially used for culinary purposes, pharmaceutical, nutraceutical, perfume, and incense industries (Pathirana and Senaratne 2020).

While the *C. zeylanicum* bark consists of cinnamaldehyde, cinnamyl acetate, cinnamic acid, cinnamyl alcohol, and β -Phellandrene (Atish Gursale et al. 2010; Zachariah and Leela 2018; Damasceno et al. 2019) its leaf consists of eugenol, cinnamaldehyde, cinnamyl acetate, cinnamyl alcohol and linalool (Paranagama et al. 2001; Shimna et al. 2017; Liyanage et al. 2020a). *C. zeylanicum* possess a diverse chemical profile even in the seedling stage grown in greenhouse conditions (Liyanage et al. 2020b). Further, there is a considerable diversity of the chemical composition among different tissues of *C. zeylanicum*, with abundant cinnamaldehyde in bark, and eugenol is in leaves. Similarly, cinnamyl acetate is dominated in fruits, while camphor is in

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its root (Kaul et al. 1974; Rao and Gan 2014). The chemical properties of *C. zeylanicum* have challenged the typical RNA extraction procedures such as Trizol, CTAB and commercial kits.

Nevertheless, extracting high-quality RNA is essential for identifying specific biosynthetic genes involved with unique chemical composition, plant behavior in response to environmental conditions, and identification of gene-specific markers for breeding programs and for studying evolutionary mechanisms of uniqueness. Therefore, we optimized a cetyltrimethylammonium bromide (CTAB) based protocol for extracting good quality RNA from cinnamon bark and leaf at different maturity stages. This protocol was further extended to other *C. zeylanicum* tissues such as flower, stem, and roots.

As a cationic surfactant, CTAB solubilizes the plant cell walls and lipid membranes of internal organelles simultaneously (Sánchez et al. 2016; Barbier et al. 2019). Since CTAB interacts with anionic nucleic acids to reduce the yield of RNA, higher concentrations of Sodium chloride (NaCl) prevent the CTAB-nucleic acid complexes formations and generate an environment to precipitate nucleic acids but polysaccharides to persist soluble (Barbier et al. 2019). Lithium chloride (LiCl) is added for the precipitation as it efficiently precipitates RNA (Sánchez et al. 2016). As the first step, we modified the CTAB lysis buffer composition, content, and its pH to facilitate quality RNA isolation. Although the RNA extraction protocols optimized for the majority of recalcitrant woody species consist of Polyvinylpyrrolidone (PVP) and phenol (Gasic et al. 2004; Farrell 2017; Rahmani and Amraee 2020), we have excluded both in our new protocol. The optimized protocol resulted in good quality and quantity of RNA for RNA-seq experiments. More importantly, this protocol was optimized with field-collected materials.

Materials and methods

Vegetative propagated *C. zeylanicum* variety *Sri gamunu* bark and leaf samples were collected from three fields, representing different agro-ecological zones of Sri Lanka; National Cinnamon Research and Training Centre, Department of Export Agriculture, Palolpitiya (GPS coordinates: 6.0241, 80.5654), Mid country research station, Department of Export Agriculture, Dalpitiya, Atabage (GPS coordinates: 7.133162, 80.590005) and Intercropping and Betel Research Station, Department of Export Agriculture, Dampelassa, Narammala (GPS coordinates: 7.404936, 80.205481). Cinnamon peel or the bark comprises parts of the cortex and secondary phloem (Ravindran and Babu 2004). We harvested cinnamon stems at the correct commercial harvestable stage and peeled the bark using a wood chisel and scraper. A harvested stem was divided into three parts as

immature, intermediate, and mature along the stem longitudinally. Two maturity stages of cinnamon leaf were assessed based on the leaf color and collected using secateurs and scissor. Moreover, we also collected few cinnamon flowers, stem (without bark) and root samples (Fig. 1). All the collected tissues were flash-frozen in the field using 50 mL RNA-free tubes containing liquid nitrogen, transported in the same, and stored at -80°C until RNA extraction.

All the solutions, buffers, materials, and equipment used for the RNA extraction are described in detail in supplementary file 1. Cinnamon samples were ground to powder using liquid nitrogen and stored at -80°C and kept frozen until the addition of lysis buffer. Either two grams of cinnamon bark tissue or 1.5 g of cinnamon leaf tissue were used for the RNA extraction.

The original CTAB based method (Chang et al. 1993) was modified to a considerable extent (Fig. 1). The protocol has two parts; nucleic acid precipitation and RNA isolation. It was performed in two days as the first part in the evening of the first day and the second part in the morning of the next day with an overnight incubation period. CTAB extraction buffer was prepared as given in the supplementary file 01, and pH was adjusted to 6.5 using concentrated HCl. PVP is considered as an essential component in the CTAB buffer optimized protocols for plants with high polyphenol content. However, we have excluded PVP for cinnamon. Then, 2% β -mercaptoethanol (200 μL / per sample) and 0.5% W/V spermidine (50 μL / per sample) was freshly added to the CTAB buffer and it was preheated to 65°C to aid pipetting. Samples were weighed and transferred to 10 mL of preheated CTAB buffer and then incubated at 65°C for 30 min with shaking and vortexed at 10 min intervals. An equal volume of chloroform (10 mL) was added, shaken vigorously for 10 s and incubated for 10 min at room temperature with 3–5 times vortexing. The samples were then centrifuged at 5000 rpm for 10 min at room temperature and 4–5 mL of liquid phase was pipetted out to a new 50 mL centrifuge tube. Extra care is needed to pipette out only the upper layer of the lysate to avoid possible DNA and impurity contaminations (Fig. 1). If the supernatant was not a clear solution and disturbed by the debris, tubes were centrifuged for additional 5–8 min. Opaque supernatants were common for cinnamon leaf and immature bark samples, probably due to its high abundance of secondary metabolites. As the last step of the first part, $0.25 \times$ volume of 10 M LiCl (1 mL to make 2.5 M final concentration) was added to each sample followed by overnight incubation on ice.

On the following morning, tubes were centrifuged at 14,000 rpm for 20 min, to pellet nucleic acid. Pellet was washed with 1 mL of 2 M LiCl and centrifuged again for 10 min at 14,000 rpm. Next, the pellet was dissolved in 500 μL of 10 mM Tris-HCl (pH 7.5) and it was transferred to a new 1.5 mL microcentrifuge tube. Then, $0.1 \times$ of 2 M

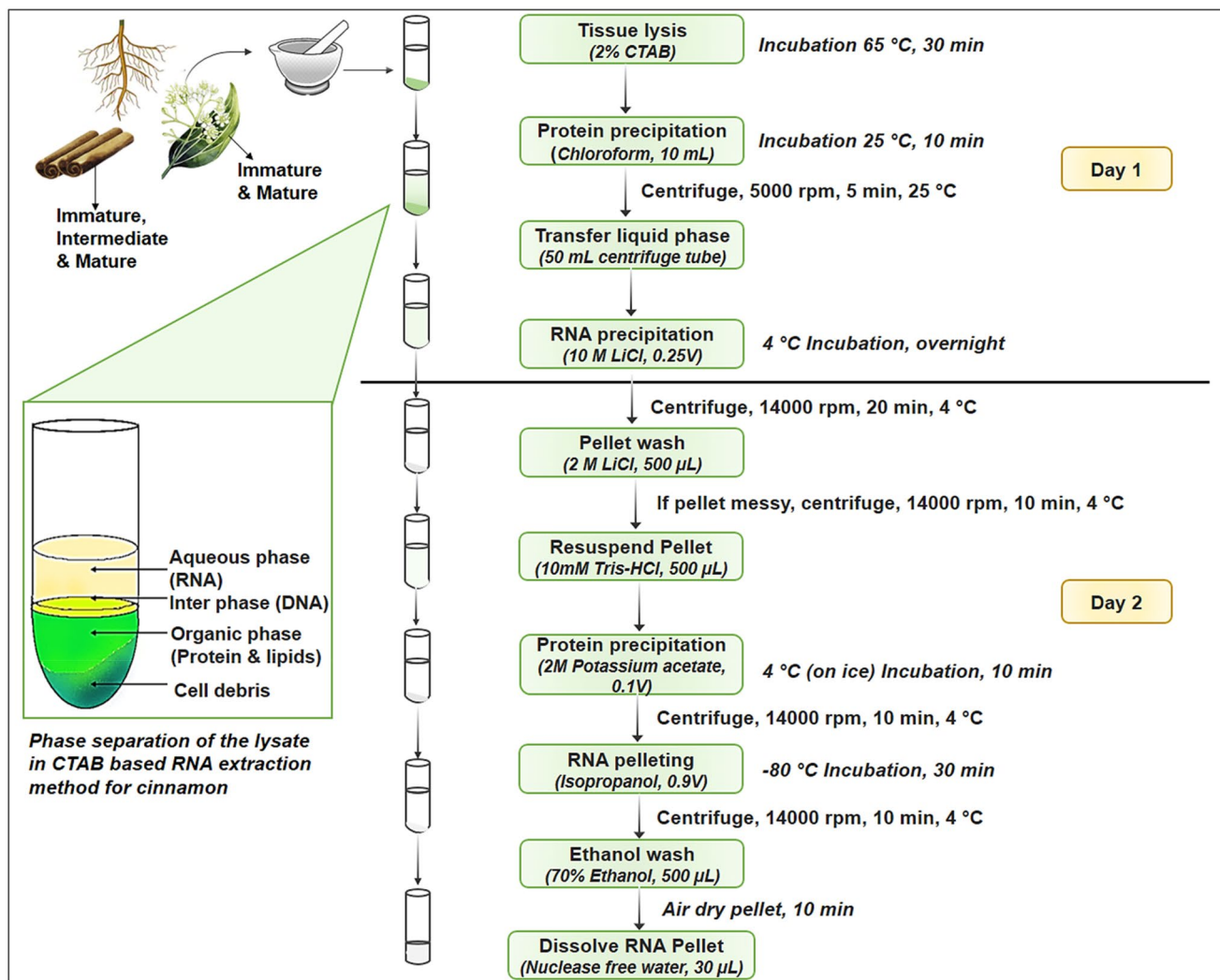


Fig. 1 Workflow of a modified RNA extraction method for cinnamon. †Cinnamon tissue types used—Bark; immature, intermediate and mature: leaf; immature and mature: inner stem: Flower: Root

potassium acetate (pH 5.5), was added, to the dissolved pellet, mixed inversely, and incubated on ice for 10 min. After centrifugation at 14,000 rpm for 10 min, the supernatant (450–500 µL) was collected into a new microcentrifuge tube. Then, 0.9 volume of cold isopropanol (about 450 mL) was added to the supernatant, mixed inversely, incubated at -80 °C for half an hour and centrifugated at 14,000 rpm for 10 min to pellet RNA. The pellet was washed with 500 µL of 70% ethanol and air-dried for 10 min. Finally, the RNA pellet was resuspended with 30 µL of nuclease-free water and stored at -80 °C in aliquots after assessing quality and quantity. RNA samples were treated with DNA free DNA removal kit (Cat. No. AM1907; Invitrogen Inc., Carlsbad, California, USA) as 0.5 µL per 1 µg of total RNA to get rid of any DNA contamination presents in samples.

The quality of the extracted RNA was determined using 1.2% agarose gel with 250 µL of sodium hypochlorite

(Aranda et al. 2012) to eliminate the RNase activity during the gel electrophoresis. Safe-Green™ dye (Cat. No. G108-G, Applied Biological Materials Inc., Richmond, British Columbia, Canada) was added to 5 µL of RNA before loading to the gel. The gels were visualized under a UV gel documentation system (Chemi Doc™ XRS + Molecular imager, Bio-Rad Laboratories Inc., Hercules, California, USA) and photographed.

The quantity and quality of the RNA samples were also analyzed using a Nanodrop spectrophotometer (Nano2000, Thermo scientific, Wilmington, Delaware, USA). The quality of the RNA was also assessed with Agilent's bioanalyzer with the Plant RNA Pico (6000) chip assay following the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA).

RNA sequencing was done at Macrogen, South Korea (Macrogen Inc., Seoul, South Korea). Libraries were

constructed using TrueSeq Stranded mRNA LT Sample Prep Kit (Cat. No. RS-122-2101, RS-122-2102, and RS-122-2103; Illumina Inc., San Diego, California, USA). Illumina sequencing by synthesis (SBS) technology was used for RNA-seq to generate paired end reads using the NovaSeq6000 platform. The raw reads were subjected to standard trimming and quality control using Trimomatic (Lohse et al. 2012). Then the quality of the sequenced reads was assessed by FastQC (Andrews 2014). The MultiQC program was used to summarize the analyzed quality results (Ewels et al. 2016).

Results and discussion

Generally, RNA extraction is more challenging than DNA extraction due to the widely present steady RNase enzyme. Nevertheless, high-quality plant RNA can be extracted if the RNase activities are inhibited (Daohong et al. 2004). There is no standard RNA extraction protocol, which suits all plants. Because, plants possess multifarious tissue types, each has specific challenges in RNA extraction compared to other organisms. Even though several protocols and commercial kits were available, high-quality RNA extraction protocols are keep updating and modifying to meet the specific challenges in plant tissues due to its diverse chemical profiles (Jaakola et al. 2001; Daohong et al. 2004; Wang et al. 2009; Kalinowska et al. 2012; de Lima et al. 2016; Maceda-López et al. 2021). The optimized CTAB based RNA extraction method resulted in good quality RNA with higher yields from all the cinnamon tissues collected from the field (Fig. 2c, Table 1).

Since cinnamon leaf and bark are the main tissue types used for commercial and medicinal purposes, we have considered cinnamon leaf and bark tissues at different development stages for the method optimization. Furthermore, inner stem, flower, and root samples were also included for completeness and expected future needs.

Removal of PVP from the CTAB buffer and adjusting its pH were the main modifications of the buffers originally described by Chang et al. (1993). In addition, the quantity of the samples, the incubation times, centrifugation time, periods, and speeds were changed in some steps. PVP is a common reducing agent in the CTAB method (Jaakola et al. 2001; Tan and Yiap 2009; Farrell 2017). While many CTAB based extraction methods consist of 1–2% of PVP (Wan and Wilkins 1994; Jaakola et al. 2001; Gasic et al. 2004; Moser et al. 2004; Ono et al. 2012; Inglis et al. 2018; Barbier et al. 2019; Rahmani and Amrae 2020) in the lysis buffer, the PVP content has increased up to 4% for fruits like maqui berry (Sánchez et al. 2016). On the other hand, cinnamon extracts are slightly sticky and slimy due to various viscous polysaccharides, and cinnamon leaf and immature

bark samples are slimier than mature bark samples. Hence, it was difficult to separate the cinnamon lysate in the first attempts with traditional Trizol, CTAB, and some commercial kits. The root, stem, and flowers were also less slimy compared to others. In slimy samples, the separation of the liquid phase at the lysis step was difficult compared to the others. The addition of PVP in the CTAB buffer intensified the slimy nature of cinnamon lysate and it was even more difficult to transfer the liquid phase after cell lysis. Moreover, PVP is responsible for take-out lots of RNA, before RNA precipitating by LiCl (Daohong et al. 2004). Consequently, DNA and partially degraded RNA was resulted from the traditional CTAB method, for all cinnamon tissue types considered (Fig. 2a). Excluding PVP from the buffer resulted in RNA bands, but with considerable DNA contaminations (Fig. 2b). A previous study on succulent plant species with high phenolic compounds and polysaccharides had shown that PVP is less effective on quality RNA extraction when high mucilage contents are present (Gehrig et al. 2000). Our results also suggest that PVP is not effective as a contaminant absorbent for plants such as cinnamon.

Further optimizations were needed to avoid slimy nature and severe DNA contaminations. The pH is an important parameter in the Trizol method (Chomczynski and Sacchi 2006). However, adjusting the pH of the CTAB buffer has not been considered in previous optimizations. Generally, DNA collects in 8–9 pH, and RNA collects in 6–7 pH. Because, in acidic conditions, RNA remains in the upper liquid phase of the lysate, while DNA and proteins remain in the interphase or lower organic phase (Chomczynski and Sacchi 2006; Sánchez et al. 2016; Farrell 2017) (Fig. 1). The unavoidable slimy nature of cinnamon led us to test the pH of the CTAB buffer and recorded around 8. Therefore, we have adjusted the buffer pH 6–6.5 by adding concentrated HCl with the objective to retain RNA in the upper aqueous phase. Lowering pH value reduced both the viscosity of the supernatant and severe DNA contaminations. The optimization step reduced the DNA contaminations in cinnamon mature bark, intermediate bark, stem, root, and flower samples, while cinnamon leaf and immature bark still had a considerable amount of DNA in extracted RNA (Fig. 2c). However, the DNase treatment resulted in no detectable DNA in RNA samples (Fig. 2d). Although many RNA extraction protocols of recalcitrant species depend on toxic and expensive phenol for the protein precipitation step, the optimized method excludes it. Instead, chloroform was efficiently precipitated proteins within samples as the modified buffer (PVP excluded) pH reduced the viscosity for a clear phase separation.

The optimized protocol was used for the extraction of 138 samples collected from the field and all of them were successful in the first attempt. It confirmed the reliability and repeatability of the protocol. The quality and quantity

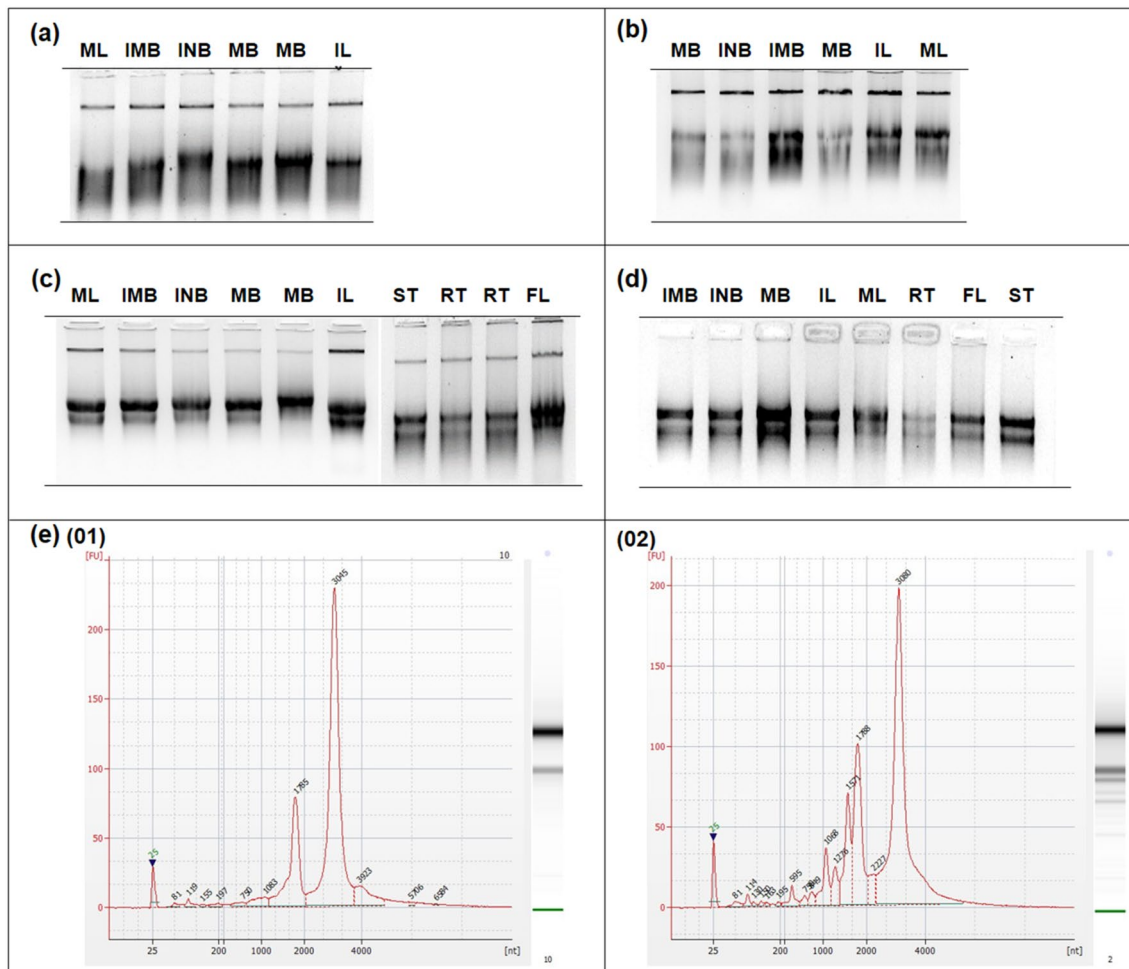


Fig. 2 RNA extracted from different cinnamon tissue types. Representative RNA gel images of Cinnamon from different maturity stages (Bark and leaf): **a** obtained by traditional CTAB method with partially degraded RNA; **b** without PVP in CTAB buffer; **c** and different tissue types after pH optimization of CTAB buffer; **d** after DNase treatment; **e** Representative bioanalyzer electropherograms of (01)

Immature bark (02) Mature leaf. RNA Gels were resolved on 1.2% Agarose gel. [†]*IMB* immature bark, *INB* intermediate bark, *MB* mature bark, *IL* immature leaf, *ML* mature leaf, *RT* root, *FL* flower, *ST* inner stem. [‡]Electropherograms were obtained from Agilent Bioanalyzer with RNA Pico chip assay (Agilent Technologies, Santa Clara, CA, USA)

Table 1 Quality and quantity measure of Cinnamon RNA

Cinnamon Tissue types	Nanodrop		[†] Bioanalyzer				
	No. of samples	Concentration (ng/μL)	260/230	260/280	No. of samples	RIN	rRNA ratio
Immature Leaf	27	2872 ± 1408	2 ± 0	1.9 ± 0.1	07	7.9 ± 0.2	5.4 ± 2.3
Mature Leaf	27	2601 ± 1331	2 ± 0	2.0 ± 0.1	19	8.0 ± 0.2	3.7 ± 0.6
Immature Bark	27	1800 ± 481	2 ± 0	1.9 ± 0.1	07	9.0 ± 0.8	2.7 ± 1.5
Intermediate Bark	27	1763 ± 503	2 ± 0	1.8 ± 0.2	06	8.4 ± 1.4	1.5 ± 1.5
Mature Bark	27	1885 ± 635	2 ± 0	1.7 ± 0.2	13	6.9 ± 2.1	2.5 ± 2.2
Inner stem	01	724	2	1.9	01	8.8	2.0
Flower	01	3523	2	2.0	01	7.9	2.0
Root	01	747	2	2.0	01	5.6	2.0

[†]Bioanalyzer readings were taken after the sample shipment, before RNA-seq by the sequencing service provider

measured with Nanodrop spectrophotometer and gel electrophoresis confirmed sufficiency for continuation (Fig. 2d, Table 1). Nevertheless, leaf and flower samples had higher concentration values while it was low in bark, inner stem, and root samples (Table 1). Variability in cellular composition and secondary metabolites in stem and roots compared to other tissue types explains the observed differences. The bioanalyzer readings qualified the suitability of selected 57 samples for RNA-seq work (Fig. 2e, Table 1). Good quality RNA-seq data validated the suitability of extracted RNA for such sensitive work (Fig. 3). The FastQC analysis indicated good phred quality with uniform high-quality base calls to the end of the forward and reverse sequence reads (Fig. 3). The phred quality is a measure of the quality of the identification of the nucleobases generated by automated sequencing (Zhang et al. 2017). This further confirms the appropriateness of the modified RNA extraction method for sensitive downstream applications. The time taken for extraction is about three hours except for the overnight incubation. We suggest starting on the evening of the first day and continuing the following morning. Nevertheless, eliminating abundant polyphenol compounds prior to the extraction as Maceda-López et al. (2021) described for polyphenolic roots may further increase the quality of Isolated RNA.

Together with its culinary value and medicinal importance (Rao and Gan 2014; Ribeiro-Santos et al. 2017; Hajimonfarednejad et al. 2019) *C. zeylanicum* is a candidate for nutraceuticals and therapeutics and many industries. However, no transcriptomic and gene expression data are available for cinnamon species except *Cinnamomum camphora* (Chen et al. 2018; Hou et al. 2020). *Cinnamomum camphora* is botanically and chemically distinct from other *Cinnamomum* species traded; *Cinnamomum cassia* Presl (Chinese cassia), *Cinnamomum tamala* Nees (Indian cassia), *Cinnamomum aromaticum* Nees (Chinese cinnamon), *Cinnamomum urmanii* Nees (Indonesian cassia) and *Cinnamomum loureirii* Nees (Saigon cassia) *Cinnamomum* (Islam et al. 2009; Li et al. 2010; Pragadheesh et al. 2013; Sharma and Rao 2014; Fajar et al. 2019). In addition, there are wild *Cinnamomum* species including several endemic in Sri Lanka are having potential future applications (Chandrasekara et al. 2021). As we identified, the sticky and slimy nature of the cinnamon is the major barrier for quality nucleic acid isolation. Since *Cinnamomum* species, in general, possess similar chemical composition and aroma to *C. zeylanicum* (Lee et al. 2015; Damasceno et al. 2019; Pandey et al. 2020) except coumarin content

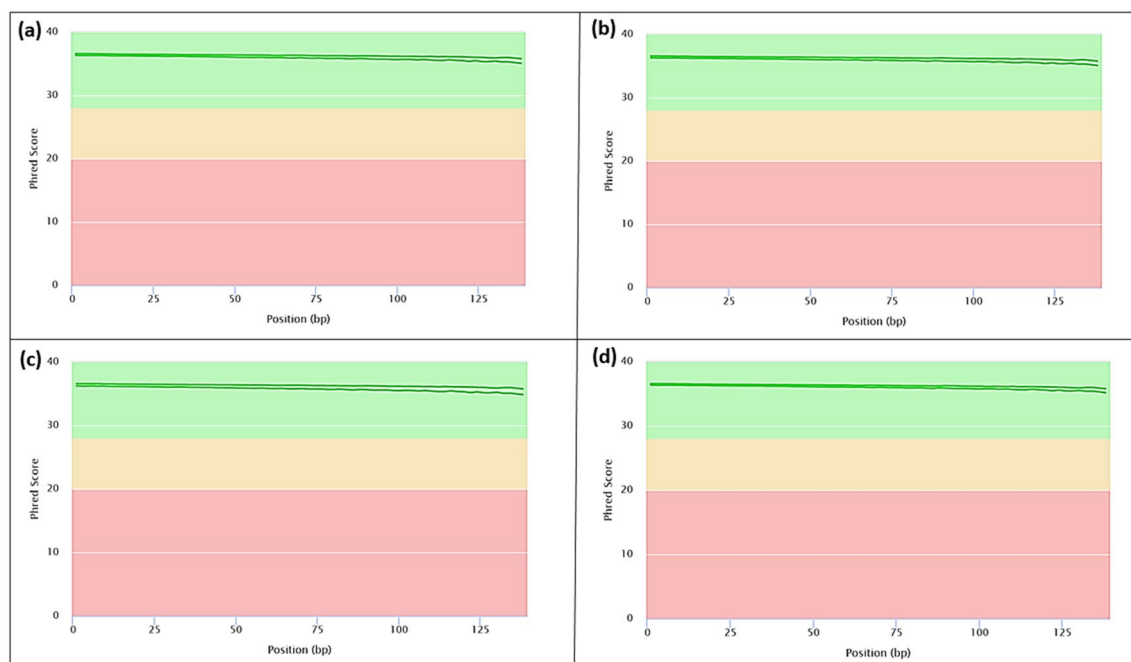


Fig. 3 Sequence quality of Cinnamon RNA extracted using the modified CTAB RNA extraction protocol. RNA was assessed by producing high-throughput RNA sequencing (RNA-seq) libraries of the different cinnamon tissue samples. The RNA-Seq libraries were pair-end sequenced (150 bp) on the Illumina NovaSeq6000 Platform. Sequencing quality assessment using FastQC version 0.10.1 (Andrews 2014) is represented in graphs describing quality across all bases from every sequence read at cinnamon **a** Bark; **b** Leaf; **c** Flower; **d** Root tissues.

†Phred quality score numerically expresses the accuracy of each nucleotide. Higher Q number signifies higher accuracy. For example, if Phred assigns a quality score of 30 to a base, the chances of having base call error are 1 in 1000. The background of the graph divides the y-axis into very good quality calls (green), reasonable quality (orange), and poor quality (red). The graphs are representative of the forward and reverse reads

(Liyanage et al. 2020a), our modifications would result in quality RNA from other *Cinnamomum* species.

Conclusion

In summary, a CTAB based RNA isolation procedure was modified to isolate high-quality RNA in sufficient quantity from different cinnamon tissues at different maturity stages. The modified protocol can reliably use for isolating high-quality RNA from different maturity stages of cinnamon bark and leaf and different tissue types such as; roots, inner stem and flowers. The extracted RNA is suitable for sensitive downstream applications such as next-generation sequencing. Overall, our method has improved purity, integrity and yield of RNA. This could be used for extracting quality RNA from field-collected materials of other *Cinnamomum* species and plants with similar chemical compositions.

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Author contributions RMMW technical assistance to RNA extraction. HMBMH Technical assistance to sample processing. PGAJ Technical assistance to sample collection, sample processing and preparing. PSAKA Technical assistance to RNA sample preparation. RAJR, YMADKY and BSB Technical assistance to sample collection. SL Assisting and troubleshooting in RNA sequencing.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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