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RESEARCH ARTICLE

A MODIFIED CTAB-BASED PROTOCOL FOR EFFICIENT ISOLATION OF HIGH QUALITY RNA FROM SECONDARY METABOLITE RICH RHIZOMES OF *CURCUMA ZEDOARIA* ROSCOE

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Abstract: Presence of high concentration of secondary metabolites in *Curcuma zedoaria* rhizomes hampers the extraction and purification of high quality total RNA, which is pre-requisite for transcriptome and gene expression studies. The main challenge is with its rhizome tissues which are rich in polyphenolics, polysaccharides and tannins, that results in poor RNA yield both in quality and quantity, due to their co-precipitation and enzymatic degradation that further limits their use in molecular processes. Thus, development of a simple, convenient and affordable approach for RNA isolation from *Curcuma zedoaria* rhizomes is necessary to facilitate its molecular studies. In the present study, four different isolation protocols were compared to access the quality and quantity of total RNA isolated from fresh and frozen rhizomes of *Curcuma zedoaria*, where a modified Cetyltrimethylammonium bromide (CTAB) based protocol resulted in distinct and intact 28 S and 18 S rRNA bands. RNA isolated from fresh rhizomes exhibited A260/A280 and A260/A230 ratios of 1.98± 0.11 and 1.94± 0.02 respectively while RNA isolated from frozen rhizomes showed corresponding ratios of 2.08± 0.08 and 2.03± 0.04. The total RNA yield obtained from fresh and frozen rhizomes was 670.1± 0.07 µg/g and 630.8± 0.09 µg/g respectively. The results demonstrated that modified CTAB method minimizes the interference of secondary metabolites and enables the recovery of high quality RNA from *C. zedoaria* rhizomes. To best of our knowledge, this the first report describing a modified CTAB based protocol optimized for RNA isolation from *C. zedoaria* rhizomes for facilitating studies related to gene expression analysis and various molecular processes.

Keywords: *Curcuma zedoaria*, Polyphenols, RNA isolation, Secondary metabolites

INTRODUCTION

Using advanced modern biotechnological tools in functional genomics is highly important for crop improvement. Identifying the underlying molecular factors that affects the plant growth and its productivity under various stress conditions and further evaluating their responses can decipher novel genes/alleles can result in stress-tolerant plants Further, decoding various stress (biotic or abiotic) tolerance mechanisms require isolation of good quality RNA, which is a crucial step in all the molecular studies. High quality RNA is also essential in developing genomic resources so as to gain the genetic information, gene expression and post-translational modifications particularly from less explored/under-utilized plant species.

Curcuma zedoaria Roscoe, also known as white turmeric, belonging to family Zingiberaceae, is native to India, China and Indonesia. Use of *Curcuma* plant rhizomes have been already mentioned in ancient Ayurvedic literature against various ailments like arthritis, asthma, diarrhea, dysentery and skin diseases, due to presence of high content of metabolites like curcumin, B turmenrone, zingiberene etc. (Gharge *et al.*, 2021). Several studies have been reported on analyzing the metabolic pathways for these important medicinal compounds using molecular tools which are primarily based on isolation of high-quality RNA (Tsouka & Masoodi, 2023). Isolation of RNA is difficult from *C. zedoaria* rhizomes due to the presence of abundant polysaccharides, polyphenols and metabolites which co-precipitate with RNA during isolation, resulting in low yield and further cause interference with reverse transcription. Though protocols for RNA isolation using CTAB (Cetyltrimethylammonium bromide), CTAB/LiCl (CTAB/Lithium chloride) SDS (Sodium dodecyl sulfate) and TRIzol method have already been reported from many plant species that are rich in polyphenols and polysaccharides (Singh *et al.*, 2024), however, these methods being expensive and time consuming do not yield good quality intact RNA due to contamination by metabolites. The methods needs to be optimized for specific target tissues, specifically for extracting RNA from under-ground tissues such as rhizomes, tubers, corms and roots which is more challenging due to high content of secondary metabolites that contaminate or degrade RNA, thus making its use inapt in further downstream applications (Mandaliya *et al.*, 2024).

The present study standardized a modified CTAB-

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based RNA isolation protocol through a comparative evaluation of four methods: TRIzol, CTAB, CTAB supplemented with LiCl, and the modified CTAB method. Modified CTAB method was proved to be efficient, reliable and cost-effective method that overcame all the limitations recorded earlier with RNA isolation from *C. zedoaria* rhizomes. Further, the isolated RNA exhibited good quality and quantity, thus making it suitable for downstream applications, including qualitative and quantitative PCR analysis.

MATERIALS AND METHODS

Plant Material and Chemicals

Fresh rhizomes of *Curcuma zedoaria* grown in the experimental fields of School of Biotechnology, University of Jammu (Jammu, India), and frozen rhizomes (*Curcuma zedoaria*) stored at -80 °C (for a minimum period of 6 months) were used for RNA isolation. The rhizomes collected from field were thoroughly washed to remove adhering soil, wrapped in aluminum foil and immediately immersed in liquid nitrogen to inhibit RNase activity and preserve the RNA integrity. Chemicals used for total RNA isolation were of molecular biology grade, thus ensuring high purity and all the required solutions were made with double-distilled water. Plastic ware and pipette tips were treated with 0.1 % DEPC-treated distilled water overnight at room temperature and autoclaved. Glassware and mortar and pestles (0.1% DEPC treated and autoclaved) were baked overnight at 140 °C in the oven.

Solutions and Reagents

RNA extraction buffer used in the study consisted of 100 mM Tris HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2% CTAB (w/v), and 2% beta-mercaptoethanol (V/V). Water-saturated phenol:chloroform:isoamyl alcohol (PCI) 25:24:1 (v/v/v), RNase-free distilled deionized water, and 100% and 75% (v/v) ethanol were also used in the study. Autoclaved double

RNA Isolation

Frozen and fresh *C. zedoaria* rhizomes were sliced into small pieces and ground into a fine powder (using liquid nitrogen), in a sterilized and pre-chilled mortar and pestle. Four different protocols were used to isolate RNA from *C. zedoaria* rhizomes, so as to identify the most efficient method for RNA isolation. The protocols used were TRIzol (Invitrogen, Carlsbad, USA) method, according to the manufacturer's instructions, original CTAB method (as described by White *et al.,* (2008); CTAB+LiCl protocol as proposed by Gasic *et al.,* (2004) and a modified CTAB protocol (method proposed by Gasic *et al.,* (2004) with modifications in few components, incubation temperature and centrifuge time). All the components for various protocols were mixed and prepared as described in table 1.

Protocol for RNA isolation as described by Gasic *et al.,* (2004) was modified (named as modified CTAB method throughout the text). 200 mg of sample (each of fresh and frozen rhizomes) was taken in 2 ml vial and 400ul CTAB buffer (preheated at 65^{o}C) was added to it and mixed well; 500ul of 2.5% Polyvinylpyrrolidone (PVP) and 200ul of 2% of beta-mercaptoethanol were also added to the CTAB buffer and the vials were incubated in water bath at 60^{o}C for 15 min. After incubation, 1 mL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) was added to the vials, and the mixture was thoroughly homogenized by pipetting. The vials were then centrifuged at 13,500 rpm for 12 minutes at 4 °C. The aqueous phase was carefully transferred to a new vial, followed by the addition of an equal volume of chloroform:isoamyl alcohol (24:1, v/v). The mixture was thoroughly mixed on ice by slow pipetting and again centrifuged at 13,500 rpm (12 minutes, 4 °C). Finally, an equal volume of chilled isopropanol was added to the aqueous phase (obtained after centrifugation) to precipitate the RNA, followed by washing the RNA pellet with ethanol (70%) and further air drying the pellet. The collected RNA pellet was dissolved in nuclease free water and stored at -80 °C. This modified protocol also included a pre-freezing step, in which freshly collected rhizomes from field were kept at -80 ^{o}C for one hour, prior to RNA isolation.

Table 1. Components of four protocols used for RNA isolation from *C. zedoaria* rhizomes

Components	Protocols			
	Original CTAB method	**Modified CTAB method**	**CTAB + LiCl method**	**TRIzol method**
Buffer	2% CTAB	2% CTAB	2% CTAB	TRIzol reagant
Tris-HCl	100 mM	100 mM	100 mM	-
EDTA	25 mM	25 mM	25 mM	-
NaCl	2.0 M	2.5 M	2.0 M	-
PVP	1 %	2.5%	2%	-
β-mercaptoethanol	5%	2%	2%	-
LiCl	4.0 M	-	10.0 M	-
Chloroform	-	-	-	0.2 vol.
Chloroform/Isoamyl alcohol	24:1	24:1	24:1	-

Phenol/Chloroform/Isoamyl alcohol	25:24:1	25:24:1	25:24:1	-
Isopropanol	2/3 vol.	2/3 vol.	0.6 vol.	Equal vol.
Ethanol	70%	70%	70%	75%
Ammonium acetate	-	-	10 mM	-
Sodium acetate	-	-	3.0 M	-

Estimation of Quantity and Quality of Isolated RNA

Purity and the concentration of isolated RNA was determined by measuring absorbance ratios of A260/A280 and A260/A230, using spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, MA, USA). Further, the integrity of the total RNA was quantified using gel electrophoresis on 1.2% (w/v) agarose gel stained with ethidium bromide and imaged using a Gel Doc system (Bio-RAD).

cDNA Synthesis from isolated RNA and its Confirmation

To remove the co-extracted genomic DNA, total RNA isolated from modified CTAB method was incubated with 2 µL of RNase-free DNase I (Fermentas, Burlington, Canada) for 30 min at 37 °C. DNase I treated total RNA (3µg) was used as a template for the first strand synthesis using Revert Aid First Strand cDNA synthesis kit (Fermentas, Burlington, Canada). The total reaction of 20 µl contained: 3 µg of total isolated RNA, 100 µM of oligo (dT) primer, 5X reaction buffer (250 mM Tris–HCl; pH 8.3; 250 mM KCl; 20 mM MgCl2; 50 mM DTT), 10 mM dNTPs, and 1 µl of RevertAid reverse transcriptase (200 units/µl). The reaction was incubated for 60 min at 42 °C, followed by 5 min at 70 °C to terminate the reaction. Synthesis of first strand cDNA was confirmed with PCR (Denaturation at 95 ^{0}C for 35 sec, Annealing at 50 ^{0}C for 40 sec, Extension at 72 ^{0}C for 30 sec for 35 cycle and final extension at 72 ^{0}C for 10 min) using primer pairs for GAPDH housekeeping gene. The sequence of primer pair used for GAPDH is Forward: 5'-GTCTCCTCTGACTTCAACAGCG-3'; Reverse: 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

RESULTS

Four different approaches viz. TRIzol method, CTAB+LiCl method, original CTAB method and modified CTAB method were used in the present study to isolate RNA from fresh and frozen *C. zedoaria* rhizomes. Using TRIzol method, A260/A280 and A260/A230 ratios of 1.05± 0.19 and 0.51± 0.20 from fresh rhizomes and 1.06± 0.21 and 0.44± 0.24 from frozen rhizomes respectively were observed in the isolated RNA. Compared to Trizol method, RNA isolated using CTAB + LiCl method, A260/A230 and A260/A280 ratios (1.59± 0.11 and 0.78± 0.16 respectively) were higher for fresh rhizomes in comparison to frozen rhizomes which was 1.37± 0.18 and 0.54± 0.13 respectively (Table 2). The low ratios in other approaches were due to co-precipitate of polysaccharides and polyphenols with endogenous RNA, hence restricting nucleotide purification. Also, varying degrees of degradation was observed in RNA samples isolated using TRIzol, original CTAB and CTAB + LiCl methods (Figure 1a,b; Figure 2a,b and Figure 3a,b). However, using a modified CTAB procedure, total RNA isolated from fresh rhizomes of *C. zedoaria* was of high quality as compared to RNA from frozen rhizomes, with distinct and intact 28 S and 18 S rRNA bands (Figure 4,b), with A260/A280 and A260/A230 ratios (1.98± 0.11 and 1.94± 0.02 respectively) for fresh rhizomes, which were observed to be much higher as compared to three other RNA isolation methods used in the present study (Table 2).

Quantity and quality of total RNA isolated from fresh and frozen *C. zedoaria* rhizomes was also compared using original CTAB method given by Gasic *et al.,* (2004) and modified CTAB approach used in the current investigation. Purity and integrity of the isolated RNA (from fresh and frozen rhizomes) was observed in both the methods, though the modified CTAB method gave better results as compared to original CTAB method (Figure 4a,b).

Using various approaches for RNA isolation, variation in yield of total RNA isolated from fresh and frozen *Curcuma* rhizomes was observed (Table 2). The quantity of the RNA extracted from fresh rhizomes using the original CTAB approach was 412.8± 0.18 µg/g tissue whereas it was 670.1± 0.07 µg/g tissue using modified CTAB protocol. For frozen rhizomes the RNA yield was 388± 0.10 µg/g tissue using the original CTAB protocol, whereas with modified CTAB method the yield increased to 630.8± 0.09 µg/g tissue. The quantity of RNA isolated from fresh rhizomes improved with the modified protocol by 1.62 folds. Overall, the modified CTAB protocol resulted in 5.31- and 2.61-folds enhancement in RNA yield as compared to TRIzol and protocol given by Gasic *et al.,* (2004) respectively for fresh rhizomes. Also as compared to frozen rhizomes, the RNA yield obtained with fresh rhizomes was observed to be more in all the four protocols under study (Table 2). Further, the isolated RNA from fresh rhizomes (using modified CTAB method) was subjected to DNase 1 treatment to eliminate any remaining traces of genomic DNA. The first strand of cDNA was synthesized and confirmed on the gel, using GAPDH as housekeeping gene (Figure 5).

Table 2. Quality and yield of total RNA isolated from *C. zedoaria* rhizomes (both fresh and frozen rhizomes)

Optical density	**RNA Quality**		**RNA Yield (µg/g tissue)**	
Original CTAB method (White *et al.*, 2008)	Fresh	Frozen	Fresh	Frozen
A260/A280	1.64± 0.25	1.48± 0.34	412.8± 0.18	388.0± 0.10
A260/A230	1.59± 0.30	1.54± 0.18	----	----
Modified CTAB method	Fresh	Frozen	Fresh	Frozen
A260/A280	1.98± 0.11	2.08± 0.08	670.1± 0.07	630.8± 0.09
A260/A230	1.94± 0.02	2.03± 0.04	----	----
CTAB + LiCl method (Gasic *et al.*, 2004)	Fresh	Frozen	Fresh	Frozen
A260/A280	1.59± 0.11	1.37± 0.18	256.3± 0.41	184.1± 0.38
A260/A230	0.78± 0.16	0.54± 0.13	----	----
TRIzol method	Fresh	Frozen	Fresh	Frozen
A260/A280	1.05± 0.19	1.06± 0.21	126.2± 0.29	118.6± 0.73
A260/A230	0.51± 0.20	0.44± 0.24	----	----

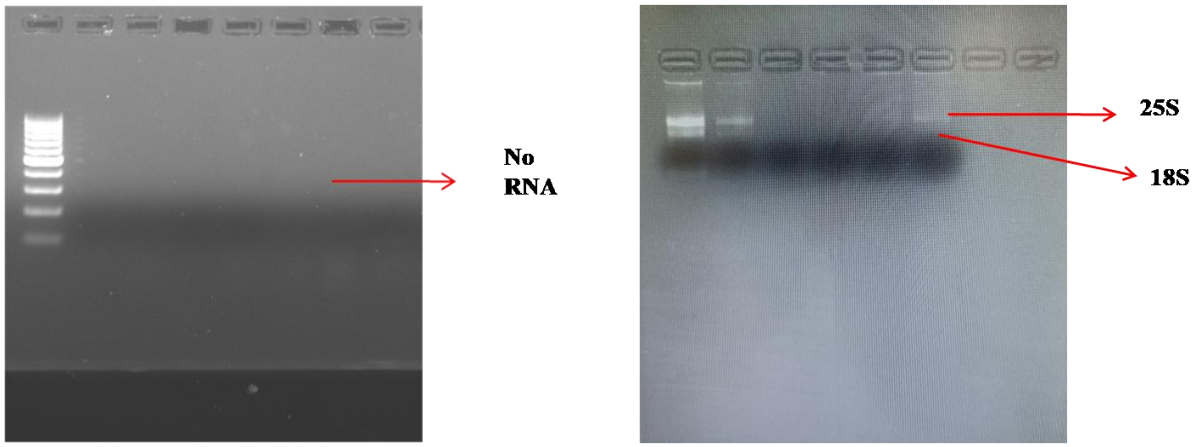


Figure 1. Total RNA isolated from a) fresh and b) frozen rhizomes of *C. zedoaria* using TRIzol method

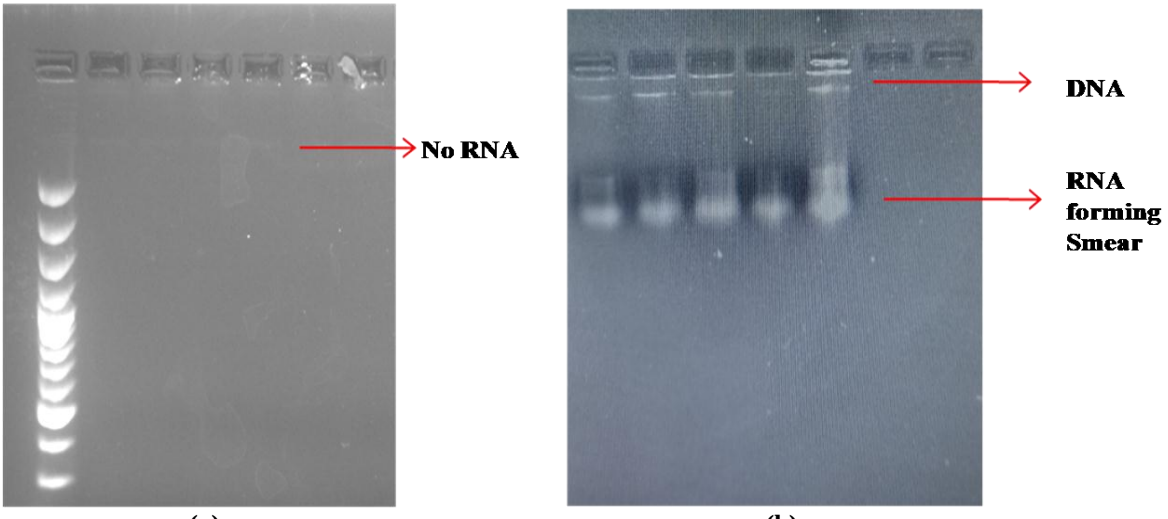
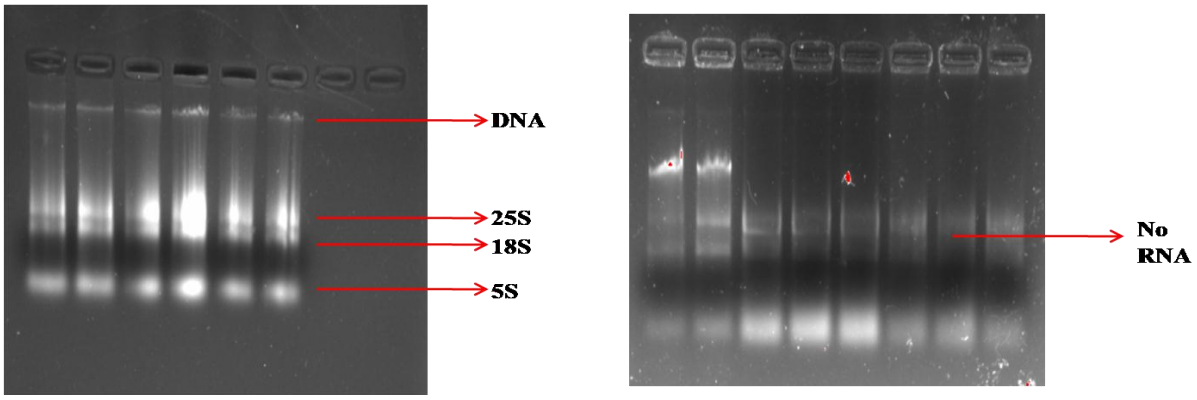
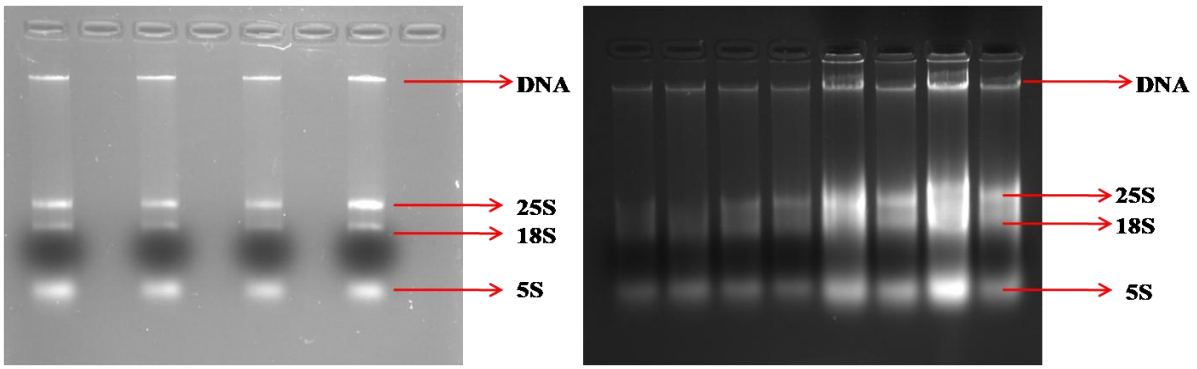


Figure 2. Total RNA isolated from a) fresh and b) frozen rhizomes of *C. zedoaria* using original CTAB method (White *et al.*, 2008)



(a) (b)

Figure 3. Total RNA isolated from a) fresh and b) frozen rhizomes of *C. zedoaria* using CTAB+LiCl method (Gasic *et al*., (2004)



(a) **(b)**

Figure 4. Total RNA isolated from a) fresh rhizomes b) frozen rhizomes of *C. zedoaria* using modified CTAB method

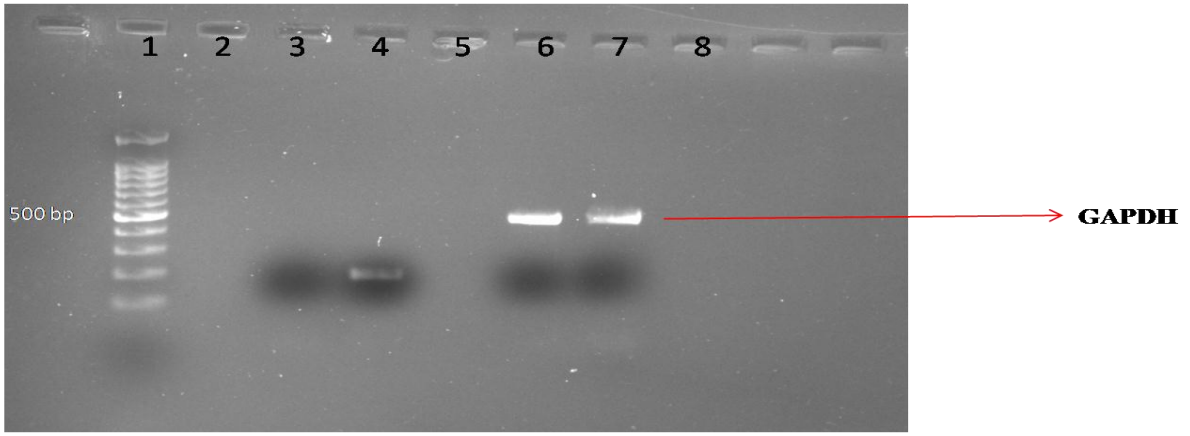


Figure 5. Confirmation of cDNA from RNA isolated from fresh rhizomes using modified CTAB method. Well no. 1: 100bp ladder, Well no. 6&7 (housekeeping GAPDH gene)

DISCUSSION

For molecular studies that involves gene expression and transcriptome analysis, isolating high-quality RNA is a crucial step. However, it is challenging to get RNA in adequate quantity and quality, particularly from those plant species whose high metabolite profile makes RNA extraction difficult and sometimes impossible. Furthermore, due to the diversity of chemical constituents contained in plant cells, there are no guidelines that are relevant for the isolation of RNA from a particular plant species/tissue (Liu *et al.*, 2018).

Presence of polyphenols, polysaccharides and alkaloids in *C. zedoaria* rhizome tissues makes it difficult to isolate good quality RNA. The polyphenolic compounds present in plant tissues undergo rapid oxidation during RNA extraction, resulting in the formation of quinones that bind to RNA and subsequently co-precipitate with it under low-ionic-strength buffer conditions. (Barbier *et al*., 2019; Wang & Stegemann, 2010).

Since the original CTAB-based RNA isolation method was modified to improve the removal of

interfering impurities, a modified CTAB protocol was developed for the efficient isolation of high-quality RNA from *C. zedoaria* rhizomes. A modified CTAB method involved protocol of Gasic *et al.*, (2004), with sight modifications where LiCl was removed from the extraction buffer in modified protocol, while LiCl at a concentration of 10.0M was used in the protocol given by Gasic *et al.*, (2004). Also, in modified protocol an increased salt concentration of 2.5M NaCl instead of 2.0M was used along with subsequent enhanced concentration of phenol:chloroform:isoamyl alcohol (25:24:1) in the extraction buffer. High ionic strength (NaCl) promotes effective partitioning of RNA away from proteins, dissolves polysaccharides and other cellular components during CTAB extraction. Since the rhizomes are rich in polyphenolics, so concentration of PVP was enhanced to 2.5% as compared to 2% used by Gasic *et al.*, (2004) protocol. This is because PVP has a strong affinity for oxidized and unoxidized phenolic compounds that precipitate in various enzymatic reactions, so PVP binds to these compounds and minimizes their interactions during RNA isolation (Wang *et al.*, 2021; Kumar & Shashank, 2025). Also, inclusion of Beta-mercaptoethanol in extraction buffer denatures RNases by breaking down disulfide bridges and disrupting their native conformation, which is necessary for enzyme functioning and further protecting the RNA from degradation.

Final precipitation with 2/3rd isopropanol also proved to be very effective. Addition of chloroform:isoamyl alcohol is important as they separate nucleic acids into a soluble phase and denatures the protein component. As reported earlier, quinones present in plant tissues bind to RNA and form high molecular-weight complexes that undergo oxidation and establish covalent linkages with polyphenolic compounds. Furthermore, polysaccharides present in plant issues also co-precipitate with RNA in presence of alcohol, which appear as contaminants during the final stages of RNA extraction and thus interfere with various molecular applications (Lee *et al.*, 2021). Additional chloroform washing steps were added in the protocol so as to remove polyphenols, polysaccharides, proteins and other unnecessary secondary metabolites. When phenolic compounds and other proteins (already present in significant amounts in the bark, rhizomes, storage roots and woody parts of plants) are eluted during the DNA/RNA extraction procedures, the quantity and quality of nucleic acid is degraded and altered. Temperature is also a primary factor in determining the integrity of RNA from plant tissues as high temperature results in RNA degradation, thus maintaining low temperature throughout RNA isolation is critical for obtaining high-quality and good yield of RNA. In the present study, all RNA isolation steps were conducted under low temperature conditions (on ice) so as to minimize its degradation and preserve integrity. This is in consistent with the observations reported by Dash, (2013) who observed brown supernatant in RNA isolated at room temperature which was due to reaction of phenolic compounds (present in plants) with the nucleic acids, while RNA isolated at 0 °C resulted in clear supernatant due to less degradation of RNA at low temperature.

CONCLUSION

In comparison to other time consuming and cost-intensive methods, the modified CTAB protocol demonstrated a significant improvement in both yield and purity of RNA isolated from fresh and frozen rhizomes of *Curcuma zedoaria*. The efficacy of this method is isolating high-quality RNA from rhizomes where other commonly used protocols often failed. Moreover, use of low-temperature conditions and optimized reagent composition effectively minimized the interference of secondary metabolites which are typically problematic in isolating high-quality RNA from plants. The reproducibility, simplicity and cost-effectiveness of this modified CTAB protocol make it highly suitable for various molecular applications. Future validation of the protocol across diverse plant species rich in metabolite will increase its applicability. This optimized protocol establishes a strong foundation for molecular techniques, particularly in plants rich in secondary metabolite content.

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CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

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