MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

A MODIFIED RNA EXTRACTION PROTOCOL FOR SECONDARY METABOLITE-RICH *AMOMUM* SPECIES (ZINGIBERACEAE)

Fathmath Shaman Fareed[1], Nallammai Singaram[1]*

[1]*School of Biosciences, Taylor's University, Jalan Taylors, 47500 Subang Jaya, Selangor, Malaysia*

*Corresponding Author: nallammai.Singaram@taylors.edu.my

History
Received: 4th July 2023
Accepted: 24th September 2023

Keywords:

Amomum; RNA extraction; Secondary metabolites; PVP; β-ME

Abstract

High-quality and pure RNA are necessary for transcriptomic studies using next-generation sequencing technologies (NGS). Peninsular Malaysian ginger species are well known for their therapeutic properties and high levels of secondary metabolites such as alkaloids, flavonoids, and aromatic compounds. The *Amomum* genus of this family is particularly well-known for its significant antioxidant and antimicrobial properties. Although many plant RNA extraction protocols have been developed in the past, the efficacy varies depending on the species and the plant and plant parts utilized. Therefore, this study aimed to compare different RNA extraction protocols and determine a protocol for the secondary-metabolite-rich ginger species of the Zingiberaceae family. RNA was extracted from fresh leaves, stems, roots, and rhizomes of *Amomum* genus using four different methods: a commercial kit, CTAB-LiCl, SDS-LiCl and SDS-Phenol. Based on the findings, the modified commercial kit produced high-quality, pure RNA for each plant tissue utilized, surpassing the other approaches. The findings of this study emphasize the importance of PVP, and β-ME in the extraction process, particularly in Zingiberaceae species with high secondary metabolites.

INTRODUCTION

The genus *Kaempferia* and *Amomum* of the Zingiberaceae family possess high medicinal values and have been widely used to treat various ailments such as stomachaches, headaches, colic, abdominal pain, flatulence, and indigestion [1, 2, 3]. This is due to the high amounts of alkaloids, flavonoids, sesquiterpenes, terpenoids and/or other secondary metabolites present in these species [4, 5]. The production of secondary metabolites in plants is influenced by the genes present, expression factors, and other regulatory proteins that are subjected to environment, age of the plant as well as the species.

The gene expression in a cell or tissue determines the type and level of active compounds present, which can be determined via RNA sequencing (RNA-seq) and RT-PCR (transcriptase polymerase chain reaction). RNA-seq provides a comprehensive view of all the RNA molecules present in a sample, including mRNA, non-coding RNA, and small RNA [6]. On the other hand, RT-PCR allows for the amplification and measurement of specific RNA molecules, providing a way to quantify the level of expression. Alternatively, microarrays allow for the simultaneous measurement of the expression levels of thousands of genes [7]. However, the success of RNA-sequencing and related analysis is subjected to the quality and quantity of RNA obtained.

The extraction of RNA from plant tissues with high levels of secondary metabolites was reported to be challenging. For example, phenolic compounds oxidize to produce quinones that can easily bind to nucleic acid, thus interfering with RNA isolation [8]. Hence, modification of RNA extraction protocols based on the species and its secondary metabolites are regularly reported.

Modified RNA extraction protocols have been developed for plant species, mainly based on the difficulties faced in obtaining high quality, quantity, and RIN (RNA Integrity Numbers). Similarly, Zingiberaceae species have been tested and proven effective with the addition of Trizol, CTAB (hexadecyltrimethylammonium bromide), or guanidinium

thiocynate (GITC). The commonly used lysis buffers such as CTAB and sodium dodecyl sulphate (SDS), are known to be effective in obtaining RNA from plants such as *Elettaria cardamomum* (Zinigiberaceae) [9] and *Dioscorea* tubers [10]. The CTAB method was first developed by Chang [11] for pine trees, which consist of high concentrations of phenolic compounds and polysaccharides. However, this method was found to be least effective for sugar beets roots, in comparison to the commercial kit method [12]. The use of SDS with the inclusion of Polyvinylpyrrolidone (PVP) and β-mercaptoethanol (β-ME) was found to be effective in RNA extraction from *Curcuma longa* (turmeric) [13], and use of Trizol and guandium thiocynate buffers were reported successful in woody plants [14] and *Plantanus acerifilia* [15] respectively.

The effectiveness of plant RNA extraction protocols is also known to be influenced by the plant and tissue type [16]. Plant species can have distinct cell wall compositions and levels of secondary metabolism, which can affect RNA extraction efficiency. This is also prominent based on the secondary wall components that can be similar or significantly different from the primary wall. One such common component is lignin, a network of phenolic compounds found in the walls of xylem vessels and fiber cells of woody tissues [17]. Various tissues are reported to have distinct levels of secondary metabolism or contaminants like pigments and polysaccharides, which can make it more difficult to extract high-quality RNA. The total flavonoid and total phenolic content of *Zingiber zerumbet* was reported to be lower in the leaves but higher in the rhizomes [18]. These compounds interfere with the extraction of high-quality RNA, and the addition of chemical agents such as PVP and β -ME in plant tissues with high levels of polyphenols, such as coffee plant tissues [19], has been reported to be effective. Similarly, [20] reported the use of PVP to increase the RNA yield in secondary metabolite-rich types of lentils.

Therefore, the development or modification of RNA extraction protocol based on the plant and tissue type is necessary, especially for ginger species that contain high levels of phenols, flavonoids, and other secondary metabolites. So, this study aims to analyse the best protocol for the secondary metabolite-rich ginger species of the Zingiberaceae family. The effect of commercial kit, CTAB-lithium chloride, SDS- lithium chloride and SDS-phenol are evaluated, along with determining the impact of tissue type on good quality and quantity of RNA.

MATERIALS AND METHODS

RNA was extracted from two species of *Amomum*: *A. uliginosum* and *A. testaceum*. Leaves, stems, roots, and rhizomes of *A. uliginosum* were collected from the Forest Research Institute of Malaysia (FRIM) and those of *A. testaceum* were collected from Batu Caves, Selangor. Voucher specimens were deposited at the national herbarium, Forest Research Institute Malaysia (KEP). The accession number for *A. uliginosum* is FRI69087 and *A. testaceum* is FRI97708.

The *Amomum* samples were washed with distilled water, pat dried, and stored in RNAlater solution (Thermofisher Scientific, U.S.A) overnight at 4˚C before being ground in liquid nitrogen. Approximately 100 mg of each sample was used for all experiments. Based on preliminary results of leaf samples (results not reported here), the commercial kit protocol was modified and tested using leaves of *A. uliginosum* and *A. testaceum*. Subsequently, the selected leaf-based protocols with minor modifications were tested to determine their efficiency in extracting RNA from different plant parts, such as pseudo-stems, rhizomes, and roots of *Amomum* species. The RNA extraction methods/protocols explored are as follows.

Commercial Kit (Macherey & Nagel NucleoSpin® RNA Plant)

Total RNA was extracted from fresh plant materials using the NucleoSpin® RNA Plant kit (Macherey Nagel, Germany). The extraction process started with the addition of 350 µl of lysis buffer RA1 and 3.5 µl (1%) β-mercaptoethanol to the pulverized plant tissues, which were vortexed vigorously. The mixture was then transferred to NucleoSpin® filter (violet ring) and centrifuged.

The filtrate was mixed with 350 µl of 70% ethanol, loaded onto a NucleoSpin® RNA plant column, and centrifuged for 30 seconds. Then 350 µl of Membrane Desalting Buffer (MDB) was added and centrifuged for another 1 minute. The flow-through was discarded, and 95 µl of DNase (1:9 of rDNase to reaction buffer) was applied directly to the silica membrane column and incubated at room temperature for 15 minutes. The silica membrane was then washed with 200 µl Wash Buffer RAW2, followed by 600 µl of Wash Buffer RA3, each centrifuged for 30 seconds, and lastly with another 250 µl of Wash Buffer RA3 and centrifuged for an additional 2 minutes. Finally, the RNA was eluted with 60 µl RNase-free water by centrifugation for 1 minute. All centrifugation steps were carried out at 25˚C and at 11,000g. The resulting RNA was stored at -20°C for further use.

Modified Commercial Kit (Macherey & Nagel NucleoSpin® RNA Plant) Protocol

The commercial kit protocol was modified based on preliminary work on leaf samples. The volume of lysis buffer, ethanol, and Membrane Desalting Buffer (MDB) was doubled and the concentration of β-Mercaptoethanol (2% β-ME) was increased. In addition, an addition of 2- 2.5% PVP during homogenization was included for roots and stems.

CTAB-LiCl Method

The CTAB (cetyltrimethylammounium bromide) [10], consisting of 2% CTAB reagent, 100mM Tris HCl buffer (pH 8.0), 2M NaCl, 25mM EDTA (pH 8.0), 2% and 2.5% PVP and 2% β – ME added just before use. PVP was used instead of PVPP used by [10], while all the other concentrations and reagents remained the same.

The plant tissue was treated with 1 mL of CTAB Buffer and incubated at 65˚C for 30 minutes, inverted at regular intervals. Then, an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed well before centrifugation for 15 minutes. Subsequently, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant, mixed well, and centrifuged to obtain two phases with the upper level being a clear supernatant. If the supernatant is not clear, the chloroform: isoamyl alcohol step was repeated (centrifuge at 12,000 g at 4˚C for 10 mins). Then, 0.25 volumes of LiCl were added to the clear supernatant in a new tube and mixed gently before storing at 4˚C overnight. The incubated supernatant was then centrifuged for 15 minutes. The pellet obtained was washed with 4M NaCl and 75% ethanol and centrifuged at 12,000g for 10 minutes. Finally, the pellet was air-dried before being dissolved in DEPC-treated water and stored at -20˚C. All centrifugation steps were carried out at 4˚C and 15,000g unless stated otherwise.

Modified SDS-LiCl Method

This protocol followed the modified SDS-LiCl (Sodium Dodecyl Sulfate- Lithium chloride) method published by [21]. The SDS extraction buffer consisted of 100 mM Tris-Cl (pH 8), 2.5M NaCl, 25 mM EDTA 2Na (pH 8), 2.5% PVP, 2.5% β-mercaptoethanol.

Approximately 800 µl – 1 mL (depending on how well the sample dissolves) of extraction buffer was added to each sample, vortexed and incubated at room temperature for 5 minutes, before adding 2% SDS, and incubated for another 2 minutes. To the collected aqueous phase, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, and vortexed. The aqueous phase was collected after centrifugation and 200 µl of chloroform was added and centrifuged. The aqueous phase was transferred to a new tube, 2M lithium chloride was added and mixed gently. Following this, 1/10 volume of 3M sodium acetate was added, mixed well, and stored at -20 ˚C overnight. The supernatant was discarded after centrifugation, and the transparent RNA pellet was washed with 2M LiCl and pre-chilled 80% ethanol. The air-dried pellet was dissolved in 35-40 µl of DEPC treated water. All centrifugation steps were carried out at 15,890g for 5 minutes at 4˚C.

SDS-Phenol Method

The SDS-Phenol buffer consisted of 2% SDS, 100mM Tris HCl. 25mM EDTA, 2% β-ME, and 2.5% PVP as described by Deepa [13]. A total volume of 2 ml of pre-warmed extraction buffer was added to the sample along with an equal volume of acid phenol: chloroform and vortexed for 10 seconds. The mixture was incubated at room temperature for 10 minutes prior to centrifugation. The supernatant was then transferred into fresh tubes, and 0.3 volume of 5M sodium acetate and 0.7 volume of acid phenol: chloroform was added and incubated on ice for 10 minutes. The supernatant obtained after centrifugation was carefully transferred to new tubes. Then, 0.1 volume of 3M sodium acetate and equal volume of isopropanol was added and incubated at -20˚C for 1 hour, followed by 10 mins of centrifugation. The pellet obtained was washed with 70% cold ethanol and centrifuged at 7,500g for 5 minutes and stored at -80° C. All other centrifugation steps were carried out at 4˚C, at 15,000g for 10 minutes.

RNA Quality Assessment

The quality and quantity of the extracted RNA was measured using UV-Visible spectrophotometer (Beckman Coulter, U.S.A). Optical density (OD) readings at 230 nm, 260 nm and 280 nm were recorded for each sample. Normally, an absorbance level $A_{260/280}$ readings of ~ 2.0 is acceptable, and $A_{260/230}$ values between 2.0 – 2.2 are considered of good purity level. Low readings may indicate low purity, which can be due to phenol residuals. The total RNA extracted were visualized on a 1% agarose gel using UV Imager (Vilber Lourmat Deutschland GmbH, Germany). Selected samples that showed good results were subjected to RIN value assessment (Apical Scientific, Malaysia). Values ranging from 1 to 10 indicate the RNA's integrity, where 1 indicates extensively degraded RNA, and 10 indicates that the RNA is completely intact.

RESULTS AND DISCUSSION

Several attempts to isolate pure and high-quality RNA from the Zingiberacea family using the protocols described above showed variable results and success rates. The commercial kit, Macherey & Nagel NucleoSpin® RNA Plant, prepared according to the manufacturer's protocol using leaf samples yielded relatively low RNA purity and concentration. However, with some modifications to the original protocol, such as doubling the lysis buffer, ethanol, MDB, increased concentrations of β-ME (2%), and addition of polyvinylpyrrolidone (PVP), RNA concentration exceeding 50ng/µl were achieved. As a result, the modified kit protocol

was used to test its efficacy for the remaining samples.

All four methods used for RNA extraction from *Amomum* samples showed good purity levels. However, in terms of RNA concentration, SDS-LiCl resulted in low RNA concentration. One possible reason for the low RNA yield in some extraction methods is the presence of polysaccharides. During phase separation, small polysaccharide particles may be eluted into the aqueous phase and co-precipitate with the RNA resulting in a decrease in overall yield [21]. On the other hand, the SDS-Phenol method gave the highest RNA concentration but the standard deviations between replicates were very high, indicating poor reproducibility. However, using the modified commercial kit protocol and the CTAB-LiCl methods, concentration above 50ng/µl were obtained, which is considered a good RNA concentration. The purity of the RNA obtained was also above 1.9 based on $A_{260/280}$ and $A_{260/230}$ readings, with only minor variations between these two methods (Table 1).

Table 1. Quantity and quality of RNA extracted from leaf tissues using different protocols

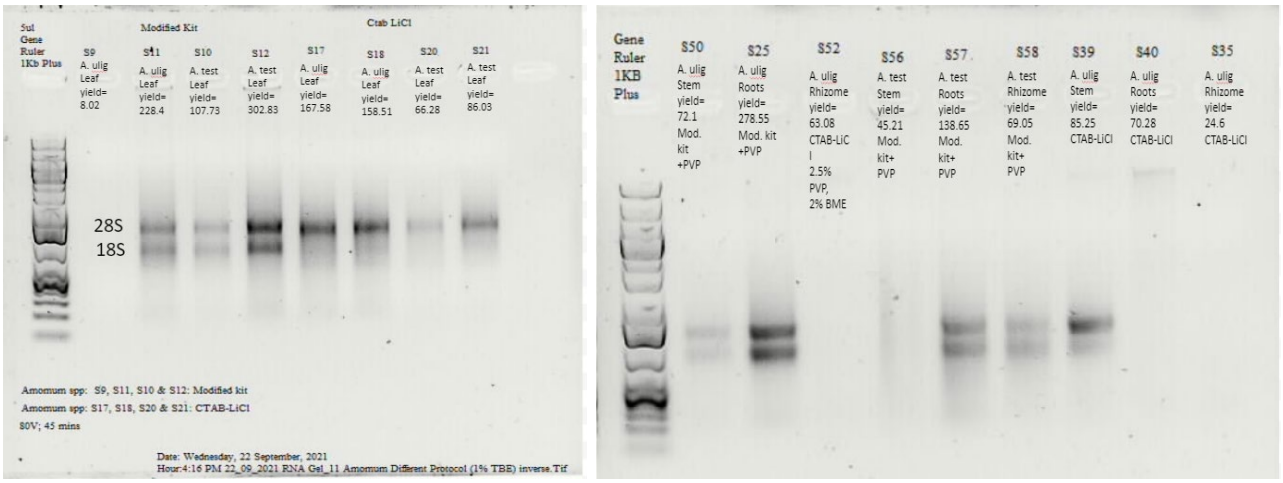
	Sample	A260/230	A260/280	Yield(ng/ul)
Modified Commercial Kit	*A. testaceum*	1.97±0.16	1.90±0.24	153±132.19
	A. uliginosum	2.04±0.02	1.97±0.14	180.79±86.60
CTAB-LiCl (2% PVP & 2 % B-ME)	*A. testaceum*	1.99±0.04	2.264±0.03	70.44±13.98
	A. uliginosum	1.95±0.04	2.05±0.06	163.05±6.41
SDS-LiCl	*A. testaceum*	1.85±0.31	1.97±0.64	8.46±4.77
	A. uliginosum	1.84±0.03	2.11±0.57	14.53±11.26
SDS-Phenol	*A. testaceum*	1.93±0.16	2.08±0.13	943.45±210.68
	A. uliginosum	1.45±0.25	1.49±0.46	238.03±159.85

To further evaluate the efficacy of the modified commercial kit protocol, the CTAB-LiCl protocol, and the SDS-Phenol protocol, they were tested on pseudo-stems, roots, and rhizomes of *Amomum* species. The modified commercial kit protocol showed good quality and quantity of RNA when the PVP concentration was increased to 2.5%. However, except for the SDS-Phenol method (with high standard deviation), all other protocols showed low purity and insufficient RNA yield for rhizomes (Table 2). This might be because Zingiberaceae family rhizomes are known to contain a higher amount of monoterpene hydrocarbons [22] that precipitate or bind to RNA [13], resulting in low RNA purity and yield. Therefore, none of the extraction protocols provided good results for rhizome plant tissues. The CTAB-LiCl protocol did not yield sufficient amounts of highly purified RNA for either pseudo-stems or roots when tested with 2% and 2.5% PVP concentrations. In contrast, the SDS-Phenol method yielded the highest amount of RNA for all three plant tissues tested, with a purity level above 2.1 for stem tissues. Previous studies by Leh [23] suggest that $A_{260/280}$ values above 2.2 may indicate possible protein contamination. Effective removal of contaminants during the phenol-chloroform extraction step is critical, otherwise the extraction may become contaminated with proteins. In addition, the presence of high concentrations of secondary metabolites, pigments, and complex polysaccharides, that may co-precipitate with RNA during precipitation or transfer over into the aqueous phase during the extraction process can be a problem. For example, during phenol-chloroform extraction, the highly pigmented and hydrophobic chlorophyll can carry over into the aqueous phase and cause contamination.

Preliminary results showed that the modified protocol of the commercial RNA extraction kit yielded distinct 25S rRNA and 18S rRNA bands on a 1.0 agarose gel, with no detectable genomic DNA (gDNA) contamination, as shown in Figure 1. This indicates that the RNA extracted by this method is of good quality and suitable for downstream applications such as library preparation for NGS. However, other methods such as the CTAB-LiCl and SDS-Phenol methods, showed limitations in terms of RNA quality and purity. The CTAB-LiCl method resulted in only a single prominent band (25S rRNA) on the agarose gel despite a relatively high RNA concentration. In contrast, the SDS-Phenol method resulted in 25S rRNA and 18S rRNA bands on the agarose gel, but with gDNA contamination, as shown in Figure 2. Contamination with gDNA can interfere with RNA-based analyses, such as RNA-Seq, as it can lead to biased results and misinterpretation of gene expression values [24]. Therefore, additional steps were included in the SDS-Phenol protocol, including treatment with DNase I to remove gDNA contaminants, proteinase K to remove any protein contaminants [25], and the addition of sodium chloride to the extraction/lysis buffer to separate nucleic acid from contaminants [26]. These modifications of the SDS-Phenol protocol successfully removed the gDNA and protein contaminants, but the procedure was time-consuming, tedious and the success rate was low.

Table 2. Quantity and quality of RNA extracted from stems, roots, and rhizome tissues of two *Amomum* species with different protocols

	Tissue	Sample	A260/230	A260/280	Yield(ng/ul)
Modified Commercial Kit (2% B-ME and 2.5% PVP)	Stem	*A. testaceum*	1.94±0.07	1.25±0.45	42.4 ±25.68
		A. uliginosum	2.06±0.5	2.03±0.22	142.76±150.61
	Roots	*A. testaceum*	2.08±0.03	2.02±0.16	204.09±139.83
		A. uliginosum	2.03±0.11	1.75±0.50	152.53±113.60
	Rhizome	*A. testaceum*	2.4	1.58	69.05
		A. uliginosum	1.89±0.17	1.08±0.45	31.25±29.01
CTAB-LiCl (2% PVP & 2 % B-ME)	Stem	*A. testaceum*	1.77±0.21	1.80±0.12	23.59±21.66
		A. uliginosum	1.90±0.03	1.87±0.08	72.78±11.34
	Roots	*A. testaceum*	1.33±0.18	0.82±0.30	12.67±9.63
		A. uliginosum	1.07±0.10	0.40±0.30	30.39±35.68
	Rhizome	*A. testaceum*	1.58±0.10	1.21±0.36	9.18±6.69
		A. uliginosum	1.17±0.05	0.35±0.11	16.84±11.50
CTAB-LiCl (2.5% PVP & 2 % B-ME)	Stem	*A. testaceum*	1.59±0.15	2.06±1.79	5.08±0.82
		A. uliginosum	1.74±0.15	1.67±074	16.73±13.08
	Roots	*A. testaceum*	1.29±0.35	0.48±0.35	13.27±11.44
		A. uliginosum	1.01±0.18	0.24±0.05	20.53±4.64
	Rhizome	*A. testaceum*	1.51±0.06	1.15±0.59	15.91±23.12
		A. uliginosum	1.11±0.06	0.25±0.04	33.90±25.71
SDS-Phenol	Stem	*A. testaceum*	1.99±0.13	2.32±0.19	1037.41±189.11
		A. uliginosum	2.13±0.11	2.33±0.06	1234.61±179.84
	Roots	*A. testaceum*	1.73±0.14	2.01±0.07	637.56±203.45
		A. uliginosum	1.95±0.06	1.98±0.12	975.39±122.70
	Rhizome	*A. testaceum*	1.97±0.25	2.25±0.16	1096.33±432.70
		A. uliginosum	1.83±0.27	2.16±0.52	645.46±276.18



A **B**

Figure 1. A: Gel image of leaf sample RNA extracts using modified kit and CTAB LiCl method. B: Gel image of stem, roots and rhizome sample RNA extracts using modified kit and CTAB LiCl method.

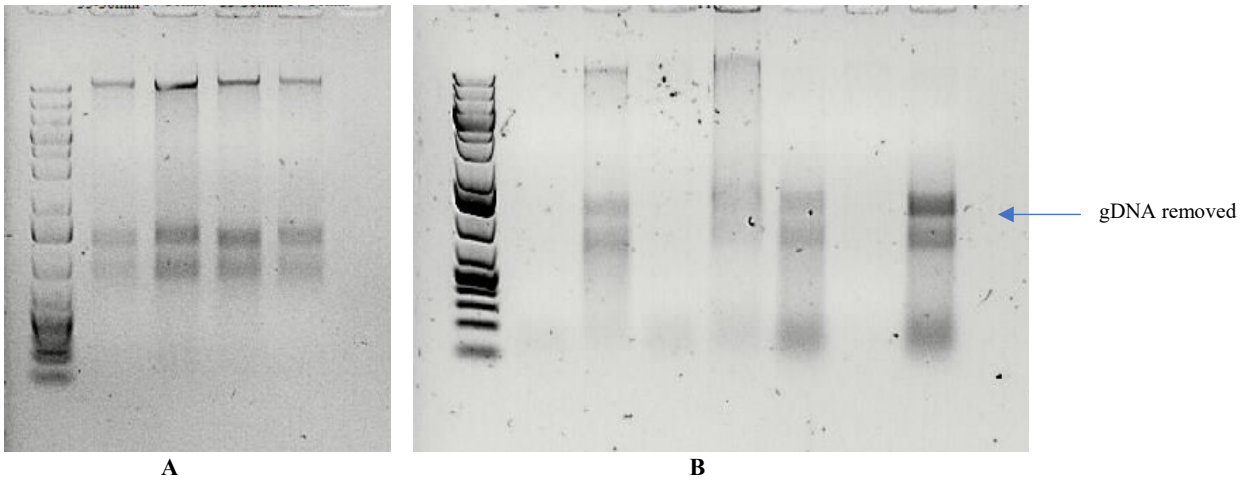


Figure 2. Gel image of leaf sample RNA extracts using SDS Phenol method before (A) and after (B) Dnase I treatment.

The results of preliminary RIN value determination of selected samples are shown in Table 3. The *A. testaceum* root sample (A12-1) which had the highest RIN value (9.3), was extracted using the modified protocol of commercial extraction kit. The leaf samples of *A. uliginosum* (S17) extracted by the CTAB-LiCl method had the lowest RIN value (2.7), which was due to possible RNA degradation. The RNA content in the root sample of *A. testaceum* (F18-T2) extracted by the SDS-Phenol method was not sufficient to determine the RIN value. The RIN values of the remaining samples ranged from 5.5 to 7.5. RNA samples with an RNA integrity score of 7 or higher are considered suitable for next-generation sequencing [27]. RIN scores greater than 7 have been reported in the past to be desirable for sensitive applications such as RNA-Seq, although RNA with RIN scores as low as 5.4 has also been used for RNA-Seq of plant tissues [23, 28]. In previous studies, RNA with RIN value of 6.5 and above has also been used for RNA-seq [29, 30]. According to [23], RIN values of 5.4 are considered suitable for RNA-Seq because plants have a wide range of rRNA sizes due to plastid ribosomes, which makes it difficult to evaluate the RIN value. In addition, it is said that the RIN value was originally developed to evaluate the RNA integrity of mammalian tissues.

Table 3. RNA integrity number (RIN) obtained for the selected samples

Sample Name and Number	Extraction Method	RIN
A uliginosum leaf S11	Modified commercial kit	6.80*
A uliginosum leaf S17	CTAB-LiCl	2.70
A. uliginosum Stem S12	Modified commercial kit	7.50*
A. uliginosum Stem F4-T2	SDS-Phenol	7.50*
A. uliginosum Stem A7-2	Modified commercial kit	7.10*
A. uliginosum Roots S25	Modified commercial kit	7.80*
A. testaceum leaf S10	Modified commercial kit	6.60*
A. testaceum Stem A10-3	Modified commercial kit	6.60*
A. testaceum Stem A10-4	Modified commercial kit	7.30*
A. testaceum Roots A12-1	Modified commercial kit	9.30*
A. testaceum Roots F18-T2	SDS-Phenol	N/A
A. uliginosum roots A44-3	Modified commercial kit	7.1*
A. testaceum leaf A43-4	Modified commercial kit	6.8*
A. uliginosum stem A41-4	Modified commercial kit	6.9*

* = RIN value 6.3 and above

The modified commercial RNA extraction kit protocol proved to be the most efficient method of RNA extraction for this project, yielding pure and high yields. Modifications such as doubling the lysis buffer in the commercial kit helped dilute the polyphenols [31]. This approach was supported by previous studies such as that of Lal [32] on RNA isolation from tea leaves with high phenolic content. The addition of PVP and β-ME is commonly used in RNA extraction, where PVP aids the removal of polyphenols by forming a complex with polyphenols through hydrogen bonding, and β-ME, a reducing agent, inactivates RNAse [32]. In this case, the addition of increased concentrations of PVP and β-ME together with the doubled commercial kit lysis buffer helped to avoid oxidation of polyphenol, resulting in improved RNA yield and purity. According to Lal [32], high concentrations of polyvinylpyrrolidone (PVP) and β-mercaptoethanol (β-ME) can help in the removal of impurities such as polysaccharides, proteins, and phenols, prevent the oxidation of phenolic compounds and result in high-quality RNA. Increased concentrations of PVP and β-ME in combination with the doubled lysis buffer in the commercial kit likely contributed to the improved RNA yield and purity obtained in this study. In *Arabidopsis*, canola, and soybean seeds [33] and maqui berry [28], which contain large amounts of storage reserves such as oil, carbohydrates, and proteins, the use of increased concentrations of PVP and β-ME was reported to help remove impurities such as polysaccharides, proteins, and phenolics and prevent oxidation of phenolic compounds, resulting in high RNA quality.

CONCLUSION

When used with the suggested modifications, the commercial Macherey & Nagel NucleoSpin® RNA Plant kit offers a reliable and efficient technique for isolating high-quality and high-yield RNA from plant tissues. The resulting RNA shows good purity and is free of genomic DNA contamination, with $A_{260/280}$ and $A_{260/230}$ ratios that are usually between 2.0 and 2.2, respectively. RNA obtained using this kit exhibits high integrity, as evidenced by RIN values consistently greater than 6.3, making it well-suited for downstream applications such as RNA-Seq and transcriptomics. The kit is also user-friendly and offers a quick and easy approach to RNA extraction, making it suitable for high-throughput applications.

In conclusion, this study has shown that RNA extraction from plant samples can be challenging, and that the type of extraction method used can significantly impact the quality and quantity of RNA extracted. Our results demonstrate that the modified commercial kit protocol was the most efficient method for RNA extraction from *Amomum* (Zingiberaceae) samples, yielding high quality and quantity of RNA. The modifications, such as doubling the lysis buffer, increasing the concentration of PVP and β-ME resulted in the avoidance of polyphenol oxidation and an improved RNA yield and purity. The findings of this study are consistent with previous studies that have reported the effectiveness of using PVP and β-ME to aid in the removal of contaminants like polysaccharides, proteins, and phenols, preventing oxidation of phenolic compounds, and resulting in high-quality RNA. Future studies can be done on other genus of Zingiberaceae family to test the effectiveness of the modified kit protocol. This study provides valuable insights into the challenges and opportunities associated with RNA extraction from plant samples and offers a practical solution for researchers working with *Amomum*.

ACKNOWLEDGEMENTS

This research was financially supported by Fundamental Research Grant Scheme (FRGS) from the Ministry of Education (MOE) FRGS/1/2019/WAB07/TAYLOR/02/1. A special thanks to Dr. Tam Sheh May for initiating and obtaining this grant.

CONFLICT OF INTEREST

There is no conflict of interests regarding the publication of this manuscript.

REFERENCES

1. Xia, Y.M., Kress, W.J. and Prince, L.M. (2004) Phylogenetic analyses of Amomum (Alpinioideae: Zingiberaceae) using ITS and matK DNA sequence data. Systematic Botany, 29(2), pp.334-344.
2. Lamxay V. (2011) The Genus Amomum (Zingiberaceae) in Cambodia, Laos and Vietnam: Taxonomy and Ethnobotany, with Special Emphasis on Women's Health (Doctoral dissertation, Acta Universitatis Upsaliensis)
3. Leong-Škorničková J and MF Newman. (2015) Gingers of Cambodia, Laos & Vietnam. Singapore Botanic Gardens, National Parks
4. Azhar, S.Z.A, Ghani, K.A., and Yusuf, N.A. (2019) Histological Observations of Adventitious Root Derived from in vitro Plantlet and Shoot Bud of *Boesenbergia rotunda* (Zingiberaceae). Pertanika Journal of Tropical Agricultural Science, 42(2).
5. Baharudin, M.K.A., Hamid, S.A. and Susanti, D. (2015) Chemical composition and antibacterial activity of essential oils from three aromatic plants of the Zingiberaceae family in Malaysia. Journal of Physical Science, 26(1), p.71.
6. Kukurba, K.R. and Montgomery, S.B. (2015) RNA sequencing and analysis. Cold Spring Harbor Protocols, 2015(11).
7. Sekhon, R.S., Briskine, R., Hirsch, C.N., Myers, C.L., Springer, N.M., Buell, C.R., de Leon, N. and Kaeppler, S.M. (2013) Maize gene atlas developed by RNA sequencing and comparative evaluation of transcriptomes based on RNA sequencing and microarrays. PloS one, 8(4), p.e61005.
8. Ding, Z.S., Jiang, F.S., Chen, N.P., Lv, G.Y. and Zhu, C.G. (2008) Isolation and identification of an anti-tumor component from leaves of *Impatiens balsamina*. molecules, 13(2), pp.220-229.
9. Palani, S.N., Elangovan, S., Menon, A., Kumariah, M. and Tennyson, J. (2019) An efficient nucleic acids extraction protocol for *Elettaria*

cardamomum. Biocatalysis and Agricultural Biotechnology, *17*, pp.207-212.

10. Barman, P., Choudhary, A.K. and Geeta, R. (2017) A modified protocol yields high-quality RNA from highly mucilaginous Dioscorea tubers. 3 Biotech, 7(2), pp.1-5.
11. Chang, S., Puryear, J. and Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. Plant molecular biology reporter, 11(2), pp.113-116.
12. Nouayti, F., Tahiri, A., Madani, I., Blenzar, A. and Lahlali, R. (2018) Comparison of RNA extraction methods for the detection of BNYVV rhizomania virus from roots of sugar beet. Comptes Rendus Biologies, 341(6), pp.343-348.
13. Deepa, K., Sheeja, T.E., Santhi, R., Sasikumar, B., Cyriac, A., Deepesh, P.V. and Prasath, D. (2014) A simple and efficient protocol for isolation of high quality functional RNA from different tissues of turmeric (Curcuma longa L.). Physiology and Molecular Biology of Plants, 20(2), pp.263-271.
14. Jordon-Thaden, I.E., Chanderbali, A.S., Gitzendanner, M.A. and Soltis, D.E. (2015) Modified CTAB and TRIzol protocols improve RNA extraction from chemically complex Embryophyta. Applications in plant sciences, 3(5), p.1400105.
15. Li, Z., Liu, G., Zhang, J., Zhang, J. and Bao, M. (2008) Extraction of high-quality tissue-specific RNA from Londonplane trees (*Platanus acerifolia*), permitting the construction of a female inflorescence cDNA library. *Functional Plant Biology*, *35*(2), pp.159-165.
16. White, E.J., Venter, M., Hiten, N.F. and Burger, J.T. (2008) Modified Cetyltrimethylammonium bromide method improves robustness and versatility: The benchmark for plant RNA extraction Vol. 3, No. 11, pp. 1424-1428.
17. Chaffey, N., Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2003) Molecular biology of the cell. 4th edn.
18. Ghasemzadeh, A., Jaafar, H.Z., Ashkani, S., Rahmat, A., Juraimi, A.S., Puteh, A. and Muda Mohamed, M.T. (2016) Variation in secondary metabolite production as well as antioxidant and antibacterial activities of Zingiber zerumbet (L.) at different stages of growth. BMC complementary and alternative medicine, 16(1), pp.1-10.
19. Paula, M.F.B.D., Ságio, S.A., Lazzari, F., Barreto, H.G., Paiva, L.V. and Chalfun-Junior, A. (2012) Efficiency of RNA extraction protocols in different types of coffee plant tissues.
20. Dash, P.K. (2013) High quality RNA isolation from ploy phenol-, polysaccharide-and protein-rich tissues of lentil (Lens culinaris). 3 Biotech, 3(2), pp.109-114.
21. Vennapusa, A.R., Somayanda, I.M., Doherty, C.J. and Jagadish, S.K. (2020) A universal method for high-quality RNA extraction from plant tissues rich in starch, proteins and fiber. *Scientific reports*, 10(1), pp.1-13.
22. Huong, L.T., Sam, L.N., Giang, C.N., Dai, D.N. and Ogunwande, I.A. (2020) Chemical composition and larvicidal activity of essential oil from the rhizomes of Amomum rubidum growing in Vietnam. Journal of Essential Oil Bearing Plants, 23(2), pp.405-413.
23. Leh, T.Y., Yong, C.S.Y., Nulit, R. and Abdullah, J.O. (2019) Efficient and high-quality RNA isolation from metabolite-rich tissues of *Stevia rebaudiana*, an important commercial crop. Tropical life sciences research, 30(1), p.149.
24. Healey, A., Furtado, A., Cooper, T. & Henry, R.J. (2014) Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. Plant
25. Li-Jie, M.A., QIAO, J.X., KONG, X.Y., WANG, J.J., XU, X.M. and HU, X.P. (2016) An improved method for RNA extraction from urediniospores of and wheat leaves infected by an obligate fungal pathogen, Puccinia striiformis f. sp. tritici. Journal of Integrative Agriculture, 15(6), pp.1293-1303.
26. Li, J.H., Tang, C.H., Song, C.Y., Chen, M.J., Feng, Z.Y. and Pan, Y.J. (2006) A simple, rapid and effective method for total RNA extraction from Lentinula edodes. Biotechnology letters, 28(15), pp.1193-1197.
27. Ahmed, R., Hossain, M.S., Haque, M.S., Alam, M.M. and Islam, MS. (2019) Modified protocol for RNA isolation from different parts of field-grown jute plant suitable for NGS data generation and quantitative real-time RT-PCR. African Journal of Biotechnology, 18(27), pp.647-658.
28. Sánchez, C., Villacreses, J., Blanc, N., Espinoza, L., Martinez, C., Pastor, G., Manque, P., Undurraga, S.F. and Polanco, V. (2016) High quality RNA extraction from Maqui berry for its application in next-generation sequencing. SpringerPlus, 5(1), pp.1-7.
29. Chen, Z., Tang, N., Li, H., Liu, G. and Tang, L. (2020) Genome-wide transcriptomic analysis during rhizome development of ginger *(Zingiber officinale* Roscoe.) reveals hormone and transcriptional regulation involved in cellulose production. Scientia Horticulturae, 264, p.109154.
30. Foong, L.C., Chai, J.Y., Ho, A.S.H., Yeo, B.P.H., Lim, Y.M. and Tam, S.M. (2020) Comparative transcriptome analysis to identify candidate genes involved in 2-methoxy-1, 4-naphthoquinone (MNQ) biosynthesis in *Impatiens balsamina* L. Scientific reports, 10(1), pp.1-17.
31. Ghangal, R., Raghuvanshi, S. and Sharma, P.C. (2009) Isolation of good quality RNA from a medicinal plant seabuckthorn, rich in secondary metabolites. Plant Physiology and Biochemistry, 47(11-12), pp.1113-1115.
32. Lal, L., Sahoo, R., Gupta, R.K., Sharma, P. and Kumar, S. (2001) RNA isolation from high-phenolic tea leaves and apical buds. Plant Molecular Biology Reporter, 19(2), pp.181-181.
33. Kanai, M., Mano, S. and Nishimura, M. (2017). An efficient method for the isolation of highly purified RNA from seeds for use in quantitative transcriptome analysis. JoVE (Journal of Visualized Experiments), (119).