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Standardization of an efficient protocol for isolation of RNA from *Cuminum cyminum*

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ABSTRACT

Cumin is one of the oldest seed species and second most popular after black pepper. Till date molecular work into cumin has been principally related to studying diversity based on phenotypic, biochemical and molecular aspects. Molecular aspects in cumin are restricted to DNA marker. But for more advanced study with profundity it is to bring forth the understanding related to transcriptome level studies. The prerequisite for such sophisticated strategy is high quality RNA. Here, we had attempted different RNA extraction procedures for fulfilling the basic preconditions for such studies. In this study we have used five different protocols to achieve high quality RNA from cumin. The RNA was isolated from root and shoot tissues using different extraction methods *viz.* Trizol method, CTAB Method, Quiagen RNAesy plant mini kit, QIAsymphony (Direct RNA extraction machine) and Phenol: choloroform method. Quality and quantity were assessed using Nanodrop [for quantity (ng/μL) at A260/280 and A260/230)], Qiaxcel [for RNA integrity score (RIS)] and 2% agarose gel electrophoresis (for intactness). RNA was converted into cDNA and visualized on agarose gel followed by real time PCR analysis to conform the quality of RNA. Eventually, the phenol chloroform extraction method was found to be most efficient for RNA extraction in terms of high yield and good quality.

1. Introduction

Cumin (*Cuminum cyminum* L.) is one of the most valuable and an important agriculture commodity in spice world and belongs to *Apiaceae* family. It is originated in Mediterranean and Near Eastern regions. Globally, it is under cultivation in Iran, Tazikistan, Uzbekistan, Morocco, Turkey, India, Egypt, Syria, Cyprus Mexico, Bulgaria and Chile (Bharti et al., 2018). India is the largest producer and consumer of cumin seed in the world (Hasan et al., 2016). Cumin seeds are used as spice due to its distinct aroma. Cumin seed contains 2.5–4.0% cuminol (volatile oil), which is responsible for the aroma and its medicinal distinctiveness. The essential oil of the seeds contain cumin aldehyde (p-isopropyl-benzaldehyde, 25%–35%), perilla aldehyde, cumin alcohol, α-dipentene, p-cymene and β-phellandrene. Conventionally, it is used to treat dyspepsia, jaundice and diarrhoea and its seeds have stomachic, astringent diuretic, stimulant, carminative and abortifacient properties (Lodha and Mawar, 2014). It is an important input material for various industries like foods, beverages, liquors, etc. (Kumar et al., 2015). In view of the enormous economic significance, it is essential to decipher inherent molecular level information for understanding its gene expression.

Highly pure and intact RNA is required for various downstream applications like Northern blotting, RT-PCR, cDNA library construction RNA interference and sequencing (Wink, 2006; Deepa et al., 2014). Generally, RNA extraction approaches involve three critical steps: solubilization (through disruption of cells or tissues in solution), deproteinization (*i.e.*, removing protein and sometimes DNA from RNA, also called extraction), and recovery of RNA free from other contaminants (Doyle, 1996). The measures for quality controls which are essential for RNA are: its purity, integrity and quantity, characterized by the 260/280 ratio having 2.1 for pure RNA, but ranges from 1.8 to 2.0 with many protocols (http://biomedicalgenomics.org/index.html). Therefore, RNA integrity and quality are considered as crucial criteria for various RNA based studies. A

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poor RNA quality leads to misleading information due to variable results during expression studies (Vermeulen et al., 2011).

Extraction of highly pure RNA especially from plant tissue is complicated due to high content of secondary metabolites (alkaloid, terpenoids, phenoles), low nucleic acid concentration (high water content), large quantity of polysaccharides compounds, fibrous tissues (such as lignin). All these biomolecules could co-precipitate and/or bind to RNA consequently poor and variable yield of RNA (Gasic et al., 2004; MacRae, 2007; Djami-Tchatchoua and Straker, 2011). Among various components, polyphenol binds irreversibly with proteins and nucleic acids which leads to oxidation and degradation of nucleic acid and makes it unsuitable for further analysis (Malnoy et al., 2001; Gasic et al., 2004; MacRae, 2007). For plant tissues, plentiful protocols are available for RNA extraction such as Trizol method, CTAB and SDS method (Yang et al., 2008), SDS-Tris saturated phenol method (Ghawana et al., 2011), SDS-acid phenol based method (Hou et al., 2011), phenol chloroform based (Chomczynski and Sacchi, 2006), activated charcoal mediated RNA extraction (Rajakani et al., 2013) and commercial kits (Plant RNeasy kit, Qiagen) (Deepa et al., 2014). None of these RNA extraction methods have been attempted in cumin. Hence, no information is available for suitable RNA isolation protocol in cumin. Keeping this in view, a work was carried out to develop an efficient methodology for RNA isolation that can give high yield and good quality total RNA from root and shoot tissues of cumin.

2. Materials and methods

2.1. Plant material

Cumin seeds (*var.* GC4) were obtained from Seed Spices Research Centre, Sardar Krushi Nagar Dantiwada Agricutural University, Gujarat, India. Seeds were grown in tray containing coco pit and kept in green house under controlled condition at Anand Agriculture University, Gujarat. Nearly 20 days old seedlings were collected for RNA extraction from root and shoot of cumin plants. Prior to RNA isolation, the samples were properly washed with distilled water followed by instantaneously frozen in liquid nitrogen (−196 °C). Another set of samples were stored in RNAlater (Ambion) and kept in deep freezer (−70 °C) for use in future.

2.2. Sample preparation

A total of five different RNA extraction protocols *viz.,* Trizol method, CTAB method, Plant RNeasy kit (Qiagen), QIAsymphony machine (direct RNA extraction) and Phenol-choloroform method were used in this study (Table 1). All the materials used during RNA extraction process were treated with a 0.5% diethylpyrocarbonate solution (DEPC) to inactivate RNases and all solutions were prepared with distilled, autoclaved and RNase-free water. Root (100 mg) and shoot (100 mg) samples were crushed separately with liquid nitrogen in pre-chilled mortar and pestle to recover a fine powder. The fine powder was collected in eppendorf tubes and further processed with different protocols for isolation of RNA. Finally RNA pellet was air dried, dissolved in nuclease free water and stored at −70 °C.

2.3. RNA isolation methods

2.3.1. Trizol method

Isolation of RNA was followed by adding 500 μL of cold (4 °C) Trizol reagent into eppendorf tubes containing tissue powder. Tubes were incubated for 5 min at room temperature followed by centrifugation for 2 min at 12,000 rpm at room temperature (RT). Supernatant was transferred to fresh tube and 100 μL 5 M NaCl was added to supernatant followed by proper mixing. A volume of 300 μL chloroform was added and mixed well by vigorous shaking. After an incubation for 5 min at RT, tubes were centrifuged at 12,000 rpm for 15 min at 4 °C. Supernatant was transferred to new tube and equal volume of isopropylacohol was added and incubated for 10 min at RT. The mix was centrifuged at 12,000 rpm for 15 min at 4 °C. Supernatant was decanted and 500 μL of 75% ethanol (ETOH) was added to dissolve the pellet. It was centrifuged twice at 12,000 rpm for 1 min at RT.

2.3.2. CTAB method

The CTAB-based method used in standardization study was a slight modification of the method reported earlier by Smart and Roden (2010) and Jamalnasir et al. (2013). The fine tissue powder was suspended in 1000 μL of pre-warmed (65 °C) CTAB extraction buffer (100 mM Tris (pH 8.0), 2% (w/v) CTAB, 30 mM EDTA (ethylene-diamine-tetraacetic acid), 2 M NaCl, 2% polyvinyl pyrrolidone-10) and 2% β-mercaptoethanol and mixed thoroughly. It was incubated in waterbathat 65 °C for 40 min with gentle mixing of solution after every 10 min. A centrifugation was performed at 14,000 rpm for 15 min at RT. Supernatant was collected and placed on ice and an equal volume of Choloroform: Isoamylalcohol (C:I) was added. The blend was gently mixed, followed by centrifugation at 13,000 rpm for 15 min at 4 °C. The aqueous phase was transferred into a new micro-centrifuge tube, and C:I step was repeated. The aqueous phase was collected and precipitated with one third volume of 8 M LiCl and incubated overnight at 4 °C, followed by centrifugation at 16,000 rpm for 30 min, 4 °C. Supernatant was removed and pellet was washed with 200 μL70% ETOH, followed by centrifugation at 13,000 rpm for 10 min at 4 °C.

2.3.3. Plant RNeasy kit (Qiagen)

The protocol was started with addition of 450 μL RLT Buffer to the powdered sample followed by was vigorous vortexing (RNeasy® Plant Mini Kit, cat. No. 74904). Lysate was transferred

Table 1
Summary of RNA extraction methodologies used in the study.

RNA extraction methods	Basis and format	Starting material	Extraction buffer	Elution buffer
Trizol	Solution based; selective precipitation of RNA	100 mg	1000 μL trizol solution	500 μL of 75% ethanol
CTAB	Solution based; selective precipitation of RNA	100 mg	1000 μL buffer (2% CTAB, 2 M NaCl, 30 mM EDTA, 2% PVP, 100 mMTrisHCl pH 8.0	200 μL of 70% ethanol
Plant RNeasy kit (Qiagen)	Silica membrane binding; Spin column format	100 mg	450 μL RLT buffer	500 μL RPE buffer
QIAsymphony Machine	Silica based RNA purification with the convenient handling of magnetic particles	100 mg	1000 μL RLT buffer plus	RNase-free water
Phenol chloroform extraction	Solution based; selective precipitation of RNA	100 mg	2000 μL buffer (10% SDS, 3 M NaOAc, 0.5 M EDTA, Saturated phenol, 100 mMTrisHCl	400 μL of 70% ethanol

to a QIAshredder spin column (lilac) placed in a 2 mL collection tube. Tube was centrifuged for 2 min at 13,000 rpm. The supernatant was transferred to new tube without disturbing the cell-debris. One half volume of absolute ethanol was added to the lysate and mixed immediately by pipetting. Properly mixed sample (650 μL) was transferred to RNeasy Mini spin column in a 2000 μL collection tube and tube was centrifuged for 15 s at 10,000 rpm. The flow-through was discarded. Buffer RW1 (700 μL) was added to the RNeasy spin column and centrifuged for 15 s at 10,000 rpm. The flow-through was discarded. Buffer RPE (500 μL) was added to the RNeasy spin column and centrifuged for 15 s at 10,000 rpm. Flow-through was discarded. Again Buffer RPE (500 μL) was added to the RNeasy spin column and centrifuged for 2 min at 10,000 rpm. After centrifugation, RNeasy spin column was placed on a new 1.5 mL collection tube. RNase-free water (30–50 μL) was added to the spin column membrane followed by centrifugation for 1 min 10,000 rpm and RNA was eluted.

2.3.4. Qiasymphony (Qiagen)

The crushed sample was transferred into the eppendorf tube and 1000 μL RLT and lysis buffer was added and properly mixed. Further, solution was homogenized by vortexing for 1 min. Samples were loaded into trough and trough was loaded onto machine and steps were preceded as per instrument instructions provided in QIAsymphony® RNA manual.

2.3.5. Phenol chloroform method

Fine crushed sample was preceded with addition of 2000 μL of extraction buffer (10% SDS, 3 M NaOAc, 0.5 M EDTA), saturated phenol (pH 7.0)/acidic phenol (pH 4), 100 mM Tris HCl) and 800 μL of DEPC water and again was re-crushed for 15 min. It was incubated for 5 min at RT. A volume of 200 μL chloroform was added, mixed well and incubated for 10 min at RT. It was centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatant was transferred to fresh vial and 0.6 vol of isopropyl alcohol was added to this. It was incubated for 10 min at RT. Solution was centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatant was decanted and 400 μL of 70% ETOH was added to pellet. It was centrifuged twice at 10,000 rpm for 5 min at RT (Ghawana et al., 2011).

2.4. Spectrometric analysis and agarose gel electrophoresisof RNA

The concentration and purity (A260/280 ratio and A260/230 ratio) was assessed using 1 μL of each sample in Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). The integrity of total RNA was also determined using Qiaxcel RNA integrity score (RIS) assay. RNA was also analyzed on 2% agarose gel electrophoresis and images were acquired using gel documentation machine, Alfa Innotech, USA.

2.5. cDNA synthesis and RT PCR

cDNA synthesis was carried out with high capacity cDNA transcription kit (Applied Biosystems) following manufacturer's instructions. The primers of endogenous gene EIF1a (elongation initiation factor 1a) were used for PCR amplication of cDNA. The total reaction volume of 10 μL containing 1 μL of template cDNA (20 ng), 5 μL KAPPA SYBR® FAST qPCR master mix (2x), 0.3 μL of each (2.5 μM) forward and reverse primer EIF1a (AT1G07940, Forward: TCAAGGATCTCAAGCGTGGTTATGT; Reverse: CAGCAATGT GGCAAGTGTGACAAT), 3.4 μL PCR grade water. Real time (RT) PCR was performed on thermocycler (CFX96 Biorad) with conditions as follows: denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 30 s. A melt curve was generated by heating from 65 to 95 °C to confirm that a single PCR product was amplified. Amplification products were also separated on 1.8% agarose gel containing ethidium bromide (0.5 μg/ml). Gels were visualized under UV light and digitally photographed with gel documentation system.

3. Result and discussion

In this present study, five RNA isolation techniques based on different extraction protocols were assessed. RNA isolation in different crops from various tissues has already been described (Hou et al., 2011; Deepa et al., 2014) however, no protocol has been standardized for RNA isolation from cumin. Therefore, in current experiment attempts has been made using range of protocols to isolate RNA from shoot and root parts of cumin.

Results of this experiment demonstrated that none of available protocols is suitable for RNA extraction as these were failed to extract RNA of high quality and quantity (Fig. 1; Table 2). In general practices, trizol protocol is used to isolate RNA from plant tissues (Yang et al., 2008). Hence, in current study RNA extraction was performed with trizol, but results were inconsistent. Moreover, both RNA quality and yield from both the samples could not be appropriately visualized and RIS was also low (Fig. 2; Table 2). During electrophoresis, the shearing was observed in the extracted RNA (Fig. 3).

Standard methods like CTAB, Plant RNeasy kit (Qiagen), QIAsymphony Machine (Direct RNA extraction), yielded low quantity of RNA (ranged from 25 to 500 ng/μL) in both root and shoot tissues (Table 3). With CTAB and Trizol, RIS value was 3 to 5 which was below than acceptable range (Fig. 2 and Table 2). Plant RNAesy kit yielded RNA between 200 and 500 ng/μL with good quality in terms of $A_{260/230}$ and $A_{260/230}$ ratio (Table 3). It also exhibited good quality peak data in nanodrop quantity and quality estimation and; RIS value was 7.1 and 7.9 for root and shoot, respectively. Thus, on basis of quality, RIS value and quantity data, the kit based protocol was found to be acceptable. But the electrophoresis of RNA on 2%

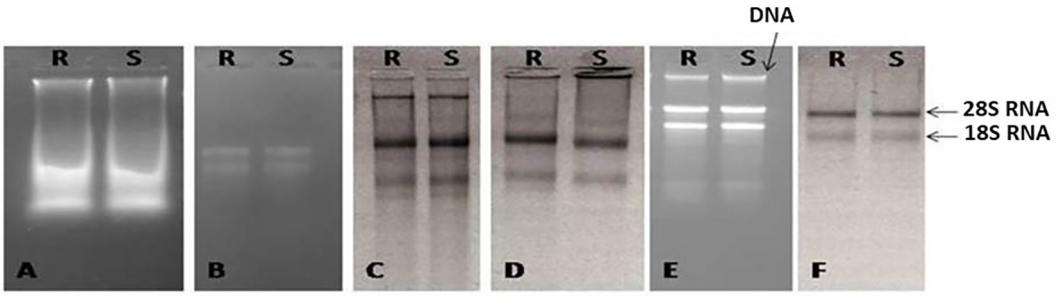

Fig. 1. Gel electrophoresis of total RNA isolated from *Cumin cyminum* using different RNA isolation methods A) Trizol method B) CTAB method C) Plant RNeasy kit (Qiagen) D) Symphony direct RNA extraction from machine E) Phenol (Tris saturated phenol) : chloroform extraction method and F) Acidic phenol : chloroform extraction method; R = root sample, S = shoot sample.

Table 2
RNA integrity score (RIS) assay data table.

Method	Sample Info	RIS	Total Concentration [ng/μl]	18 S			28 S		
				Presence	Size [nt]	Conc. [ng/μl]	Presence	Size [nt]	Conc. [ng/μl]
Trizol	Root	3.4	8199.56	Yes	1658	123.89	No	n/a	n/a
	Shoot	4.6	2975.40	Yes	1702	229.59	No	n/a	n/a
CTAB	Root	5.1	749.18	Yes	1815	124.70	No	n/a	n/a
	Shoot	5.2	371.06	Yes	2080	40.10	No	n/a	n/a
Plant RNeasy kit (Qiagen)	Root	7.1	415.65	Yes	2077	23.48	No	n/a	n/a
	Shoot	7.9	763.06	Yes	2089	15.45	No	n/a	n/a
QIAsymphony Machine	Root	6.9	900.93	Yes	2110	28.89	No	n/a	n/a
	Shoot	7.3	1502.06	Yes	2062	27.68	No	n/a	n/a
Phenol : chloroform extraction	Root	8.1	1105.27	Yes	2084	44.48	yes	4816	69.40
	Shoot	9.3	2209.25	Yes	2110	119.24	yes	4563	64.01

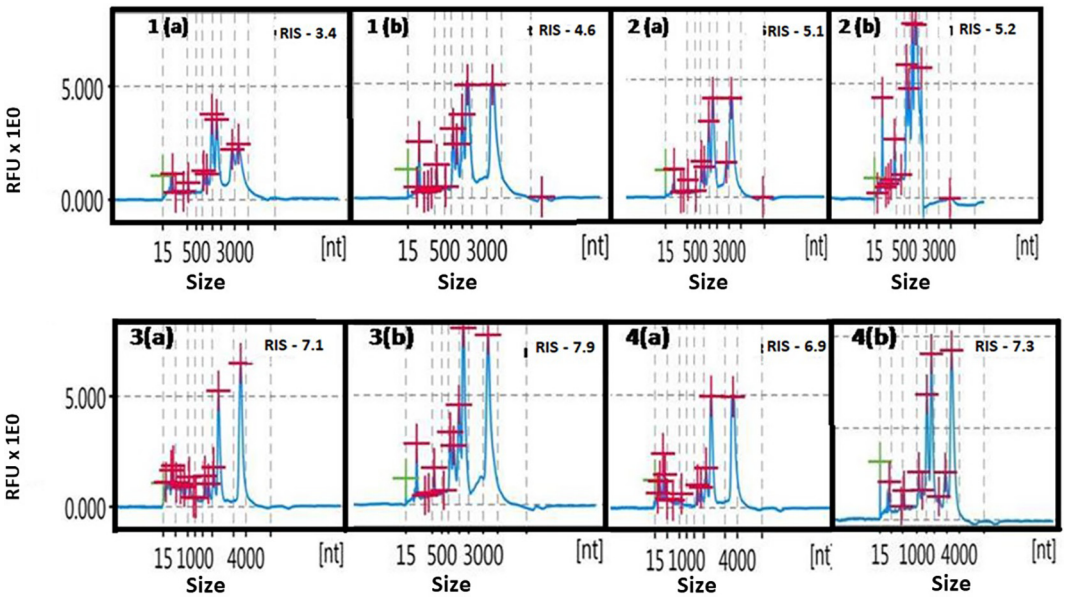

Fig. 2. Electropherogram of total RNA isolated using 1. Trizol a) root b) shoot; 2. CTAB a) root b) shoot; 3. Plant mini kit a) root b) shoot; 4. Symphony machine a) root b) shoot.

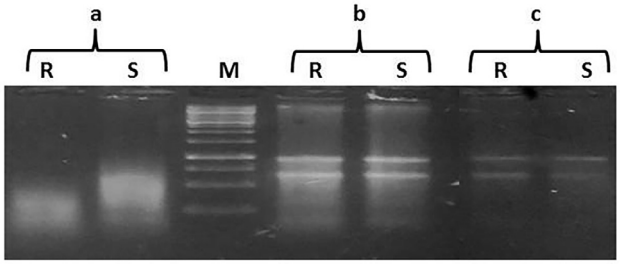

Fig. 3. DNA contamination removal strategies using a) 5 M LiCl; b) DNase and c) acidic phenol (pH 4); M = 100 bp ladder, R = root sample, S = shoot sample.

agarose gel showed very low concentration of 28S RNA as compared to 18S. The same trend of result was observed in direct RNA extraction from QIAsymphony machine.

Phenol chloroform method of RNA extraction gave maximum RNA yield (800–1400 ng/μL) from both the samples (Table 2). The A260/A280 ratio was 2.16 (shoot) and 2.04 (root) while A260/A230 was 2.26 (shoot) and 2.17 (root). Moreover, the presence of sharp band of 28S RNA compared to18S RNA during electrophoresis also indicated the recovery of intact and protein- and polysaccharide-free RNA (Fig. 1E) which confirms excellent quality of RNA. Although phenol-chloroform method yielded good quality and quantity of RNA but DNA contamination was detected. To

Table 3
Spectrophotometric analysis of RNA isolated using different protocols.

Method	Tissue sample	RNA yield (ng/μl)	$A_{260/280}$	$A_{260/230}$
Trizol	Root	252.11	2.10	0.66
	Shoot	420.71	1.80	0.96
CTAB	Root	24.02	2.08	0.97
	Shoot	25.56	1.74	0.44
Plant RNeasy kit (Qiagen)	Root	242.63	2.11	2.11
	Shoot	404.17	2.05	1.94
QIAsymphony Machine	Root	187.89	1.96	1.64
	Shoot	246.84	2.11	1.89
Phenol chloroform extraction	Root	815.5	2.16	2.26
	Shoot	1352.1	2.04	2.17

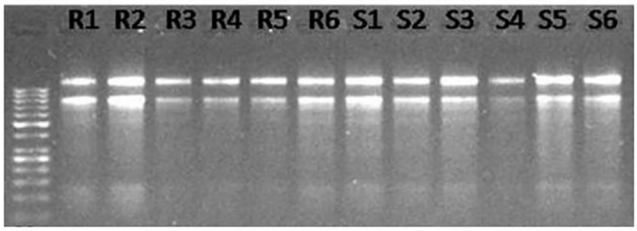

Fig. 4. RNA extraction acidic Phenol: chloroform from root [R1–R3 (control), R4–R6 (wilt infected)] and shoot [S1–S3 (control), S4–S6 (wilt infected)] samples in three replications.

overcome the problem of DNA contamination, various strategies were used 1) addition of 5 M LiCl followed by overnight incubation (Sambrook and Russel, 2001), but this time consuming strategy could not resolved the problem as traces of DNA was present (Fig. 3a), and 2) DNAase treatment, reported in various protocols (Kiefer et al., 2000; Xu et al., 2010), was carried out but this enzymatic based resolution produced smear of RNA suggesting degradation of RNA (Fig. 3b).

Finally, acidic phenol (pH 4) was used in place of saturated phenol (pH 7.0) during preparation of extraction buffer which resulted in intact, pure and high quality RNA (Fig. 3c) with RIS value 8.1 and 9.3 for root and shoot, respectively. Eventually, protocol with acidic phenol was exploited for RNA extraction for downstream process. As reported earlier, the reason for desired results is successful separation of RNA and DNA between the organic phase and the aqueous phase (Brawerman et al., 1972; Perry and Kelley, 1972). At basic pH *i.e.* 7–8, phosphate diesters get negatively charged, therefore DNA and RNA both get partitioned into the aqueous phase. However, at acidic pH, most of the proteins and small DNA fragments (<10 kb) remains into the organic phase and large DNA fragments and some proteins retains at the interphase (Chomczynski and Sacchi, 2006). In such chemical environment, due to the readily neutralization of negative charges of DNA than RNA (means DNA has higher pKa/lesser acidic than RNA) DNA acquires into phenol phase while RNA catches into aqueous phase (Bradley et al., 2001). Moreover, this acidic environment greatly reduces the RNase activity (Zhang et al., 2017) which ultimately reduced the RNA degradation. With this protocol, one could get quality RNA from both tissues with better consistency from cumin (Figs. 4 and 5). Maximum RNA yield obtained was 1352.1 ng/μL in shoot tissue.

This above protocol, comparatively, low RNA yield was recovered in root (815.5 ng/μL) than shoot tissues. This may be due to presence of high content of different polyphenols, alkaloids and polysaccharides in root in comparison to shoot. Such secondary phytochemicals are major hurdle in proper RNA isolation as reported in many plant species such as in turmeric (Deepa et al., 2014), tea (Das et al., 2013), coffee (Paula et al., 2011) and lentil (Dash, 2013). These phytochemicals especially polyphenolics oxidized rapidly and form quinones which freely binds to RNA (Loomis, 1974). Likewise, polysaccharides compound co-precipitate with the RNA in low ionic concentration buffer (Birtic and Kranner, 2006).

In addition to different quality parameter checks, a successful cDNA preparation is also a representation of good RNA quality as the reverse transcription process is liable to impurities in sample and therefore pertinent check for proper RNA quality assessment and subsequent downstream analysis such as gene expression studies is needful (Tang et al., 2007; Vasanthaiah et al., 2008). Here, we had successfully prepared cDNA from isolated RNA (root and shoot) samples of cumin. Furthermore, a successful real time PCR was carried out using primer of *EFI 1a* gene of carrot (Tian et al., 2014) resulted 154 bp product in root and shoot RNA sample (Fig. 6). The expression level of the gene is determined by Cq values, the low Cq value represents that the gene has higher expression. In the present study EIF 1A gene obtained mean Cq value of 24.44 for shoot samples and 23.69 for root samples, represented abundant expression of endogenous genes in shoot and root samples. All samples of root and shoot showed a single peak in melting curve analysis showed that there was absence of non-specific amplification (Fig. 7). Thus RNA isolated was proved to be a robust template for PCR amplification.

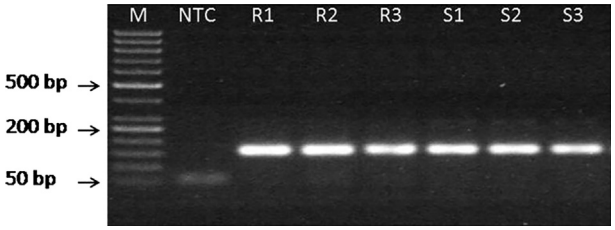

Fig. 6. Agarose gel electrophoresis of qPCR product of endogenous gene EIF 1a. Lane 1–50 bp makrer, 2-NTC, 3–5 root sample, 6–8 shoot sample.

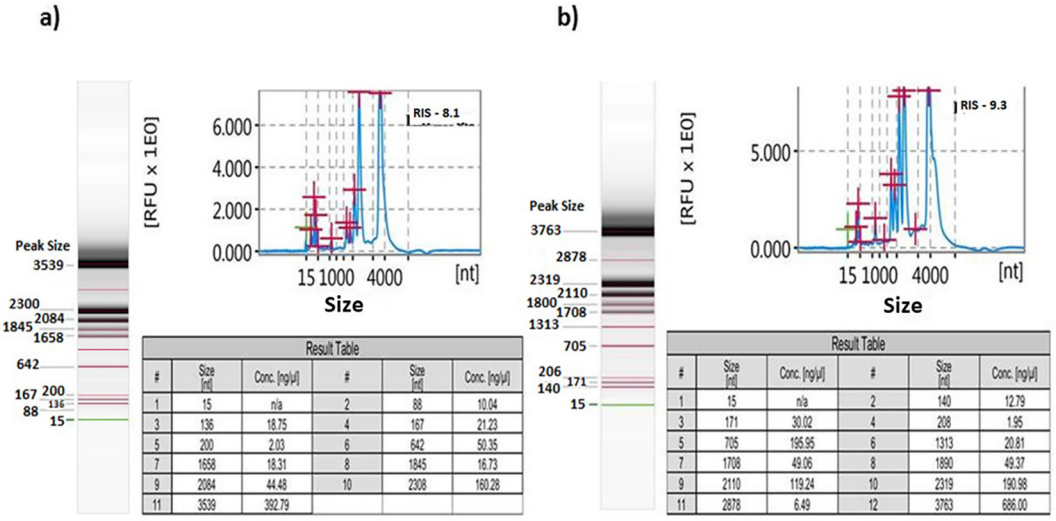

Fig. 5. Electropherogram of total RNA isolated using acidic phenol : chloroform a) root b) shoot.

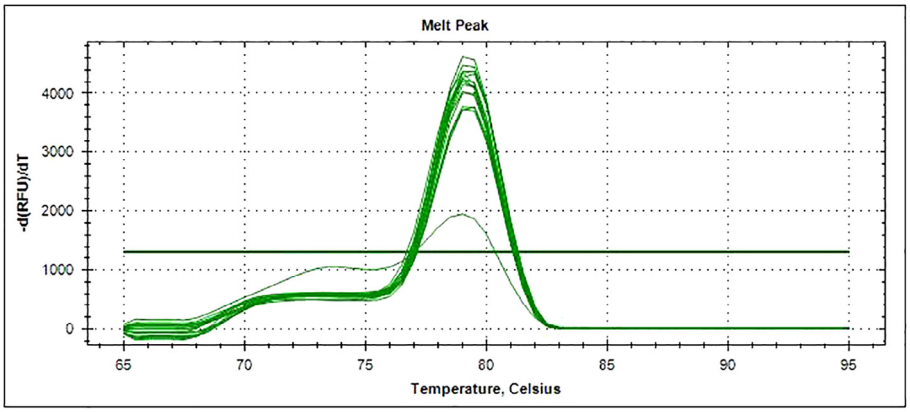

Fig. 7. Melting curve generated for EIF1a gene by qPCR.

4. Conclusion

In this study, five RNA extraction methodologies were conducted and compared for isolation of high quantity and quality RNA for different down-streaming processes such as expression analysis, transcriptome studies. Amongst all the various procedures used, phenol chloroform extraction method proved to be the most efficient in isolating RNA with high yield and good quality from cumin (*var*. GC4).

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