Malays. Appl. Biol. (2017) 46(2): 87–95

A MODIFIED CTAB-BASED PROTOCOL FOR TOTAL RNA EXTRACTION FROM THE MEDICINAL PLANT *Impatiens balsamina* (BALSAMINACEAE) FOR NEXT-GENERATION SEQUENCING STUDIES

LIAN CHEE FOONG[1], ANTHONY SIONG HOCK HO[1], YANG MOOI LIM[2] and SHEH MAY TAM[1*]

[1]*School of Biosciences, Taylor's University, Jalan Taylors, 47500 Subang Jaya, Selangor, Malaysia*
[2]*Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, Lot PT 21144, Jalan Sungai Long, Bandar Sungai Long, 43000 Kajang, Selangor, Malaysia.*
[*] *E-mail: shehmay.tam@taylors.edu.my*

Accepted 20 April 2017, Published online 27 June 2017

ABSTRACT

High RNA quality, integrity and reproducibility are essential for functional genomics study involving library preparation and sequencing using next generation sequencing (NGS) technologies. The medicinal herbaceous plant, *Impatiens balsamina,* possess a rich source of diverse bioactive compounds including polysaccharides and phenolic compounds, hindering the extraction of high quality total RNAs. Four existing RNA extraction protocols were performed using fresh leaf material of *I. balsamina*, namely the CTAB method, the TRI reagent® method, modified TRI reagent® method and a commercial kit but the results were unsatisfactory. This led to the development of a modified CTAB protocol by combining CTAB/polyvinylpyrrolidone (PVP) extraction with using commercial spin column purification for successful isolation of high quality total RNA from the leaf material of *I. balsamina,* that is suitable for NGS applications (RIN values $\geq$ 7). This new method also successfully extracted total RNA from flower buds and capsules of *I. balsamina*.

Key words: *Impatiens balsamina*, total RNA extraction, CTAB protocol

INTRODUCTION

Impatiens balsamina L. (Balsaminaceae) or garden balsam is a popular, attractive ornamental plant that also has been widely featured in traditional medicinal treatments in China, Japan, Taiwan and Thailand for various maladies such as rheumatism, dermatitis, bruises, scrofulosis and fingernail inflammation (Kang *et al*., 2013; Ding *et al*., 2008; *Dictionary of Chinese Crude Drugs*, 2003; Oku & Ishiguro, 2002). *Impatiens balsamina* is known to be a rich source of diverse bioactive compounds, such as polysaccharides (Shoji *et al*., 1994), alkaloids (Kang *et al*., 2013; Baskar *et al*., 2012), flavonols and flavonoids (kaempferol, quercetin, rutin, astragalin, nicotiflorin, naringenin and their derivatives) (Oku & Ishiguro, 2001; Hargen Jr, 1966), phenolic compounds (Nurul *et al*., 2010; Bohm & Towers, 1962) and quinones (1,4-naphthoquinone, lawsone, 2-methoxy-1,4-napthalene-1,4-dione) (Ishiguro *et al*., 1998; Little *et al*., 1948). It is therefore not surprising that modern pharmacological and food preservation studies have reported that different *I. balsamina* extracts demonstrated antipruritic and antidermatitic (Oku & Ishiguro, 2001), antibacterial (Lim *et al*., 2006), antifungal (Thevissen *et al*., 2005), antitumor and anticancer (Liew *et al*., 2015; Ong *et al*., 2015; Wang *et al*., 2011; Ding *et al*., 2008) activities.

Despite the medicinal and potentially economic importance of the natural compounds produced by *I. balsamina*, not much information is currently available about the genes and biosynthesis pathways of various secondary metabolites for *I. balsamina.* With the advent of next-generation sequencing (NGS) technologies, it is now possible

* To whom correspondence should be addressed.

to perform transcriptomic sequencing (RNA Seq) of important but non-model plant species (Ekblom & Galindo, 2011). However, NGS technologies require good concentration of high quality total RNA (e.g. 10 µg) characterized by spectrophotometer measurements of A_{260}/A_{280} ratio > 1.9 and usually RNA integrity number (RIN) score greater than 8 (though values > 6 have been accepted) as a measure of high quality sample (Romero *et al*., 2014).

Performing total RNA extraction using leaf tissues is common for plants, but often leaf material can be especially problematic due to excessive presence of secondary metabolites such as alkaloids, polysaccharides, and phenolic compounds. For example, phenolic compounds (e.g. terpenoids and tannins) are known to be readily oxidized to form quinones, which irreversibly bind covalently with proteins and nucleic acids to form high molecular complexes and hinder isolation of good quality RNA (Ding *et al*., 2008; Iandolino *et al*., 2004; Salzman *et al*., 1999).

Although there are various protocols and commercial kits available for RNA extraction from plant tissues, they may not be suitable for use on all plant species, hence, various RNA extraction protocols for recalcitrant plant species/different plant tissues have been published (Kansal *et al*., 2008; Vasanthaiah *et al*., 2008). For plant species with tissues facing difficulties in isolating total RNA such as *I. balsamina,* modifications to existing protocols is needed to overcome difficulties for successful RNA isolation. In this study, several RNA extraction methods including the CTAB method (Chang *et al*., 1993), TRI reagent® method, modified TRI reagent® method and a commercial kit were attempted for total RNA extraction from leaf material of *I. balsamina*. However, the quality and yield of the total RNA obtained using these methods did not meet the requirement for NGS. This led to the development of a modified CTAB/ polyvinylpyrrolidone (PVP) extraction protocol combined with commercial kit spin columns purification for successful isolation of high quality total RNA that is suitable for NGS applications. To the best of our knowledge, this is the first report of an RNA extraction protocol for *I. balsamina* for the purpose of Next Generation Sequencing.

MATERIALS AND METHODS

Plant materials

Three mature cultivated plants of *I. balsamina* (pink flower cultivar) were bought from a local commercial nursery (Jacky Lim's nursery, Sungai Buloh, Selangor, Malaysia). For fresh young leaves of *I. balsamina,* approximately 200 mg of leaves was collected and immediately ground into fine powder using mortar and pestle with the addition of liquid nitrogen. Afterward, the modified CTAB method developed in this study was then used to perform total RNA extraction for the flower buds and the capsules of *I. balsamina*. Flower buds with 6-8 mm were collected. Three developmental stages of the capsule were collected, i.e. in early stage (less than 10 mm in length and no clearly formed seeds), mature stage (12-18 mm in length and the seeds appear in white), and post-breaker stage (more than 20 mm in length and the seeds appear in complete brown colour). Each of the total RNA extraction methods were conducted for multiple times.

Preparation of CTAB buffer

The cetyl trimethylammonium bromide (CTAB) extraction buffer is composed of 2% hexdecyltrimethylammonium bromide (CTAB; Acros Organics, Geel, Belgium), 2% of polyvinylpyrrolidone (PVP-40; Sigma-Aldrich, St. Louis, Missouri, US), 100 mM Tris-HCl (pH 8) (Tris-Base: Sigma Aldrich, St. Louis, Missouri, USA; Hydrochloric acid fuming 37%: Merck, Kenilworth, New Jersey, US), 2.0 M sodium chloride (Sigma-Aldrich, St. Louis, Missouri, USA), and 25 mM ethylenediaminetetracetic acid (EDTA; Sigma-Aldrich, St. Louis, Missouri, USA) in RNase-free water (Bio Basic Inc., Markham, ON L3R 8T4, Canada) and sterilized using standard autoclave conditions at 121°C for 15 mins (Chang *et al*., 1993). Prior to use in RNA extraction, 2% β-mercaptoethanol (Merck, Kenilworth, New Jersey, USA) was added and the solution was warmed to 50°C in the waterbath.

CTAB method

The method followed the protocol published by Chang *et al.* (1993). Briefly, 2 ml of CTAB reagent premixed with 40 µl β-mercaptoethanol (preheated to 65°C) was added into the sample (ground to fine powder) in a 15 ml RNase-free centrifuge tube. The mixture was vortexed and mixed thoroughly, and then incubated in a 65°C waterbath for 10 min. 200 µl of chloroform:isoamyl alcohol (24:1) (Sigma Aldrich, St. Louis, Missouri, US) mixture was added, mixed well, and chilled on ice for 5 min. After that, the sample was centrifuged at 12,000 x g for 10 min. All centrifugation steps were performed at 4°C using Eppendorf Centrifuge 5415R (Eppendorf, Hamburg, Germany). The upper clear layer of the sample was transferred to a new tube and mixed with ¼ volume of 10 mol/L Lithium chloride solution (Merck, Kenilworth, New Jersey, US), and kept in the -20°C freezer overnight. Next, the sample was centrifuged at 12,000 x g for 20 min. The supernatant was removed and 500 µl of RNase-free water was added to dissolve the visible pellet, followed by the

addition of an equal volume of chloroform:isoamyl alcohol (24:1). This mixture was centrifuged at 12,000 x g for 10 min. The supernatant was transferred to a new centrifuge tube, two volumes of absolute ethanol was added and allowed to stand at -20°C for 30 min. The sample was again centrifuged at 12,000 x g for 10 min, discarding the supernatant and the pellet washed twice with 1 ml of 70% ethanol. The final total RNA pellet was air-dried in room temperature (RT) for 20 min and then dissolved with 35 ul of RNase-free water.

Modified CTAB method

The modified CTAB method was developed in this study, which successfully extracted total RNA from *I. balsamina*. Firstly, 4.5 ml of CTAB reagent (details of preparation provided in Appendix) pre-mixed with 90 µl of β-mercaptoethanol (Merck, Kenilworth, New Jersey, US) was added to the homogenized sample in a 15 ml centrifuge tube and incubated at 50°C for 5 min. Then, 1 ml of chloroform-isoamyl alcohol 24:1 (C0549, Sigma Aldrich, St. Louis, Missouri, USA) was added, mixed well and chilled on ice for 5 min. The sample was centrifuged for 10 min at 13,200 x g and the supernatant transferred to a new 1.5 ml centrifuge tube. All centrifugation steps were performed at 4°C using Eppendorf Centrifuge 5415R (Eppendorf, Hamburg, Germany). One volume of cold 70% ethanol (prepared from pre-cooled to 4°C) was added and the sample mixed well. The mixture was then transferred to a mini spin column (Geneall® Column type W from Ribospin™ Plant kit, GeneAll Biotechnology, Songpa-gu, Seoul, South Korea) and centrifuged at 10,000 x g for 30 s. The flow through was discarded, 500 µl of buffer RBW (from Ribospin™ Plant kit, GeneAll Biotechnology, Songpa-gu, Seoul, South Korea) was added to the spin column and centrifuged at 10,000 x g for 30 sec. Next, 70 µl DNase I reaction mixture (4 µl DNase I pre-mixed with 140 µl buffer DRB; Thermo Fisher Scientific, Waltham, Massachusetts, USA) was applied to the sample in the spin column and incubated at room temperature for 10 min. [This step can be skipped if no genomic DNA contamination is present, or the concentration of DNase I / incubation time changed based on amount of DNA detected]. Next, 500 µl of buffer RBW was added, incubated for 2 min and the sample was then centrifuged at 10,000 x g for 30 sec. Then, 500 µl of buffer RNW (from Ribospin™ Plant kit, GeneAll Biotechnology, Songpa-gu, Seoul, South Korea) was added to the sample, followed by centrifugation at 10,000 x g for 30 sec. The flow through was discarded and the sample was centrifuged again at 10,000 x g for an additional 1 min to remove the wash buffer and dry the silica gel membrane completely. Finally, total RNA was eluted by adding 32 µl of RNase-free water onto the center of the membrane in the mini spin column and incubated for 2 min prior to centrifugation at 10,000 x g for 1 min. The total RNA samples were stored at -80°C until further analysis. Afterward, the buds and the seeds (in three different developmental stages, i.e. in early stage, mature stage, and post-breaker stage) were extracted using the same method to investigate the versatility of the modified method. The fresh material used were 200 mg of tissue.

TRI reagent® RNA isolation method

This method was adopted from Chomczynski & Sacchi (1987). Total RNA extraction was performed using TRI reagent® according to the manufacturer's recommendations (Molecular Research Center, Cincinnati, US). The ground leaves sample was added with 1 ml of TRI reagent®, vortexed and incubated at RT for 5 min. The sample was then added with 0.2 volume of chloroform (Sigma Aldrich, St. Louis, Missouri, US), mixed vigorously for 15 sec and allowed to stand for 10 min at RT. After centrifugation at 12,000 x g for 15 min at 4°C, the upper clear layer was transferred into a new tube. An equal volume of 2-propanol (Merck, Kenilworth, New Jersey, US) was added, mixed well, and allowed to stand for 5 min at RT, followed by centrifugation at 12,000 x g for 10 min at 4°C. The supernatant was removed and the precipitated pellet retained and washed twice with 1 ml of 75% ethanol and centrifuged at 7,500 x g for 5 min, 4°C. The RNA pellet was left to air-dry and adequate volume of RNase-free water was added to dissolve the pellet before storing at -80°C.

Modified TRI reagent® method

This method was adopted from Na *et al.* (2013) with slight modification. The ground sample was added with 2 ml of ice-cold acetone (pre-cooled in -20°C) and 30 µl of β-mercaptoethanol in a centrifuge tube. The sample was then oscillated for 20 min and centrifuged at 6,000 r/min for 10 min at 4°C. The supernatant was removed and mixed with 2 ml of TRI reagent, mixed and let stand at RT for 30 min, followed by centrifugation at 12,000 x g for 10 min. The supernatant was transferred to a new tube and 0.2 volume of chloroform was added, mixed vigorously for 15 sec, and allowed to stand for 10 min at RT. The following steps were similar to TRI reagent® method.

Commercial RNA extraction kit

The Qiagen RNeasy Plant™ kit (Qiagen, Hilden Germany) was used following the manufacturer's protocol. Total RNA was eluted with 35 µl of RNase-free water.

Total RNA Assessment

The concentration and purity of total RNA was determined using a DU®730 Series UV/Vis spectrophotometer (Beckman Coulter, Brea, California, US) by the measured ratios of A_{260}/A_{280} and A_{260}/A_{230}. The total RNA extracted from samples were visualized on 1.0% agarose gel using a gel documentation system (Quantum ST5, Vilber Lourmat, Marne-la-Vallée Cedex 1 F-77202, France). Leaf samples that showed promising results using the above method were further subjected to a Bioanalyser RNA 6000 Nano chip analysis (Agilent Technologies, Santa Clara, California, US).

RESULTS AND DISCUSSION

Attempts were made to isolate total RNA from the leaves of *I. balsamina* using four conventional protocols, such as the CTAB and TRI reagent methods, as well as a commercial column based extraction kit (Qiagen RNeasy Plant^{TM} kit), but none produced sufficiently good quality RNA. The TRI reagent method obtained relatively high yield (ranged from 360.22 to 479.53 µg/ml) but low integrity of total RNA. The modified TRI reagent method (ranged from 47.14 to 123.40 µg/ml) and the Qiagen RNeasy Plant^{TM} commercial kit (ranged from 1.99 to 7.75 µg/ml) gave variable or did not yield sufficient amount of RNA whilst the CTAB method failed to produce any RNA pellet at the end of a long and tedious protocol, very little (to none) of total RNA were recovered perhaps due to loss of RNA during the multiple solvents extraction steps (Table 1, Figure 1).

In this study, a previously described CTAB method initially developed for RNA extraction of pine tree tissues characterized by abundancy of polysaccharides and phenolics contaminants (Chang *et al*., 1993), was adapted and optimized for successful extraction of high quality total RNA from *I. balsamina*. Results showed that total RNAs extracted from the leaves of *I. balsamina* using the modified CTAB method yielded high amounts of total RNA (range of 146.91 to 420.93 µg/ml) (Table 1, Figure 1). The CTAB method usually involves an initial nucleic acid extraction step, a phase separation step using chloroform, and an alcohol precipitation step. A typical CTAB extraction buffer contains CTAB (a cationic surfactant) to break the plant cell walls, polyvinylpyrrolidone (PVP) to prevent the oxidation of polyphenols in cell walls and extracellular matrices (Jaakola *et al*., 2001), sodium chloride to dissolve polysaccharides and CTAB-RNA complexes, and β-mercaptoethanol to irreversibly denature RNases (White *et al*., 2008). Key modifications in our method involved substantially increasing the volume of CTAB reagent (lysis buffer) used. This is up to five times the recommended volume of other protocols to successfully remove contaminating secondary compounds, and the incorporation of spin column purification to improve the quality of total RNA extracted and significantly shorten the protocol

Table 1. **Total RNA Quality Assessment.** Spectrophotometric determination of extracted total RNA quantity and quality from *Impatiens balsamina* fresh leaf tissues with different protocols

	Sample	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio	RNA yield (µg/ml)	Remarks
Modified CTAB method	C4(1)	2.238	2.377	194.31	
	C7(2B)	2.254	2.172	146.91	RIN = 7.5
	C7(2A)	2.458	1.834	420.93	RIN = 7.0
	C4(2)	2.242	2.299	181.59	
	C7(1A)	2.124	2.207	86.873	
	C7(1B)	2.197	2.381	145.710	
CTAB method (Chang *et al*. 1993)	C4(1) C4(2)	N.D.	N.D.	N.D.	Possible lost of total RNAs during extraction
TRI reagent® method	C5(1)	1.160	0.806	479.53	High yield but low A_{260}/A_{280} and A_{260}/A_{230} ratios
	C5(2)	1.316	0.538	360.22	
Modified TRI reagent® method (Na *et al*. 2013)	C4(1A)	1.003	0.202	95.52	Low yield, low A_{260}/A_{280} and A_{260}/A_{230} ratios
	C4(1B)	1.128	0.314	123.40	
	C4(2A)	0.933	0.146	67.34	
	C4(2B)	0.834	0.093	47.14	
Commercial kit	C4(1)	1.631	0.188	7.754	Low yield and low A_{260}/A_{230} ratios
	C4(2)	1.451	0.062	2.705	
	C4(1)	2.146	0.010	6.225	
	C4(2)	2.001	0.006	1.988	

N.D., not detectable.

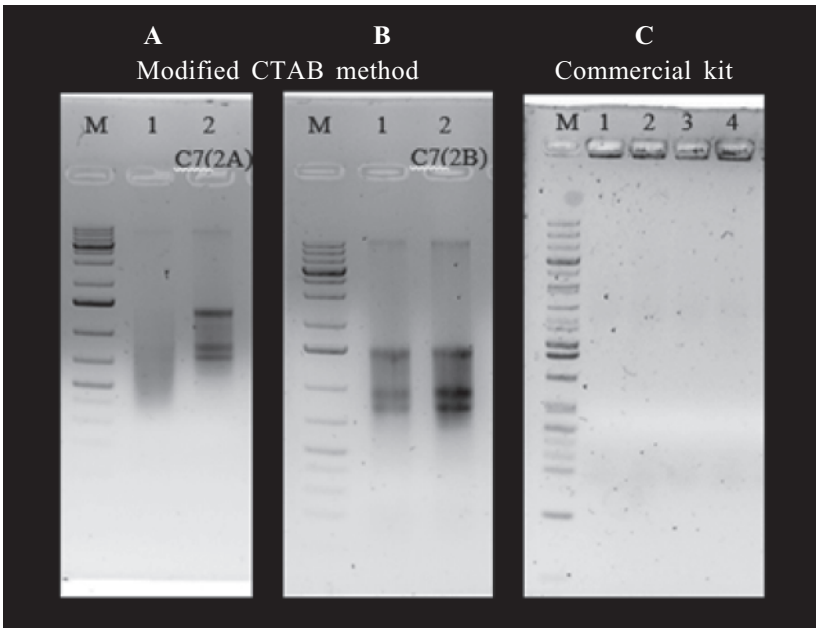

Fig. 1. Comparison of total RNAs isolated from leaf tissues of *Impatiens balsamina* using different RNA extraction protocols on 1% (w/v) agarose gel. M, Gene Ruler 10kb plus DNA ladder marker. (A) Lane 2, total RNA samples from modified CTAB method, (C7(2A), RIN 7.5); (B) Lane 1-2, total RNA samples from modified CTAB (Lane 2: C7(2B), RIN 7.0); (C) Lanes 1-4, total RNA samples from commercial kit method. Volume of RNase-free water used to dissolve/elute total RNAs were the same in all protocols.

time. The CTAB method (Chang *et al*., 1993) is complex as it involved two rounds of incubation with LiCl and long precipitation times, and the protocol required about two days to complete. In addition, a DNase treatment step was included to treat genomic DNA contamination (if present). The modified CTAB protocol is not as complicated and can be completed within two to three hours.

To evaluate RNA quality, absorption spectra maxima at 280 nm, 260 nm, and 230 nm were measured to calculate the spectrophotometric absorption ratios. Table 1 shows the RNA quality and concentration from all the evaluated extraction methods. Total RNA quality measured by spectrophotometric ratios showed that the modified CTAB method produced samples with A_{260}/A_{280} ratios higher than 1.9 (average 2.12) and A_{260}/A_{230} ratios ranging between 1.83 and 2.38, indicating that the RNA sample is relatively pure and free from proteins, polyphenols and polysaccharides contamination (Porebski *et al*., 1997). However, total RNAs obtained from the existing protocols did not meet the normal threshold readings for both the A_{260}/A_{280} and A_{260}/A_{230} ratios. For example, the A_{260}/A_{280} nm ratios ranged from 0.83 to 1.32 and 1.45 to 2.15 for RNA samples obtained by the TRI reagent® and Modified TRI reagent® methods and commercial kit respectively, indicating detectable levels of protein contamination. Similarly, the A_{260}/A_{230} absorbance ratios were lower than 0.81 to non-detectable readings in the RNA obtained by other methods, indicative of polyphenol and polysaccharide contamination. The total RNA extracted by the CTAB method did not show any spectrophotometric absorbance readings. These contaminants will potentially interfere or inhibit library preparation for NGS (Healey *et al*., 2014) as well as other molecular biology applications such as PCR, restriction digests, or DNA sequencing by inhibiting the action of Taq polymerases or restriction endonucleases (Sahu *et al*., 2012).

The quality of total RNA samples obtained from different methods was checked using 1.0% (w/v) agarose gels. The integrity of the RNA samples prepared by the modified CTAB method is evident where two sharp, intact and well-resolved bands were revealed (28S and 18S ribosomal RNA bands) (Figures 1A and 1B). For the total RNA samples extracted with TRI reagent method, no band was detected on the gel but smears were seen around the wells. Most commercial RNA extraction reagents/ kits such as TRI reagent® is an improved version of the use of acid guanidinium thiocyanate as a denaturing agent by combining phenol and guanidine thiocyanate in a mono-phase solution to inhibit RNase activity more effectively. Total RNA is separated from DNA/proteins under acidic conditions upon centrifugation (Chomczynski &

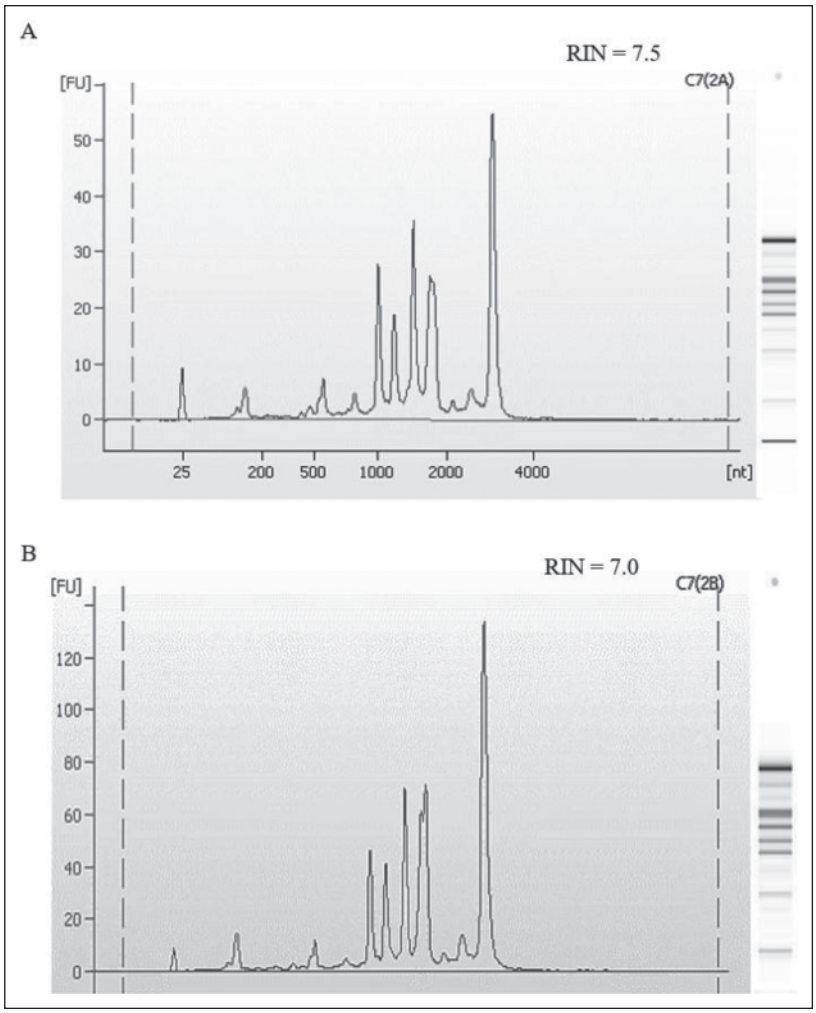

Fig. 2. Agilent Bioanalyzer electropherograms of two total RNA samples isolated from the fresh leaves of *Impatiens balsamina*. A) Sample C7(2A) has RIN of 7.5 and B) Sample C7(2B) has RIN of 7.0.

Sacchi, 1987), but in this study, the guanidine based methods (commercial kit and TRI reagent protocols) repeatedly gave low yields of poor quality total RNA (Figure 1C), possibly due to the presence of high levels of polysaccharides which were hard to be eliminated by guanidium salts. This suggests that the high yields shown by spectrophotometry were likely the result of false measurements of contaminants (Christou *et al*., 2013), most likely due to the presence of polysaccharides which tend to co-precipitate with RNAs in low ionic strength buffers (Carra *et al*., 2007; Gehrig *et al*., 2000) or in the presence of alcohols (Kansal *et al*., 2008). Small polysaccharides can partition into the aqueous phase during phase separation and co-precipitate with RNA in the precipitation step, or entrap nucleic acids to clog the silica-membrane spin column in commercial kit-based methods, hence interferes with the subsequent loading of RNA samples into the gel during electrophoresis or Northern blotting (Kansal *et al*., 2008). RNA integrity for two promising RNA samples (C7(2A) and C7(2B)) extracted by the modified CTAB method were further determined using a Bioanalyzer RNA 6000 Nano chip, and both samples have RIN values $\geq$ 7 (Table 1, Figure 3). This protocol could be applied for RNA extraction in other medicinal herbaceous plants with a similar problem, where high secondary compounds are present in plant tissues such as leaves.

To evaluate the versatility of the modified CTAB method, flower buds and three developmental stages of the capsules of *I. balsamina* were subjected to total RNA extraction using this method. Results show successful total RNA extraction using this method. The A_{260}/A_{280} and A_{260}/A_{230} ratios of these samples are above 2.0 with good concentration values (as seen in Table 2). As shown in Figure 3, visualization of total RNA using agarose gel electrophoresis reveal clear bands (28S and 18S) for the flower buds and capsule samples.

Table 2. Total RNA Quality Assessment. Spectrophotometric determination of extracted total RNA quantity and quality from *Impatiens balsamina* flower bud and capsule tissues using the modified CTAB method

Sample	A_{260}/A_{280} *ratio*	A_{260}/A_{230}*ratio*	*RNA yield (µg/ml)*
Flower bud	2.148	2.639	193.6
	2.204	3.306	110.84
Capsule (early stage)	2.173	2.315	243.77
	2.093	2.304	88.456
Capsule (mature stage)	2.259	2.476	283.11
	2.26	2.647	220.88
Capsule (post breaker stage)	2.035	2.547	206.06
	2.161	2.213	169.01

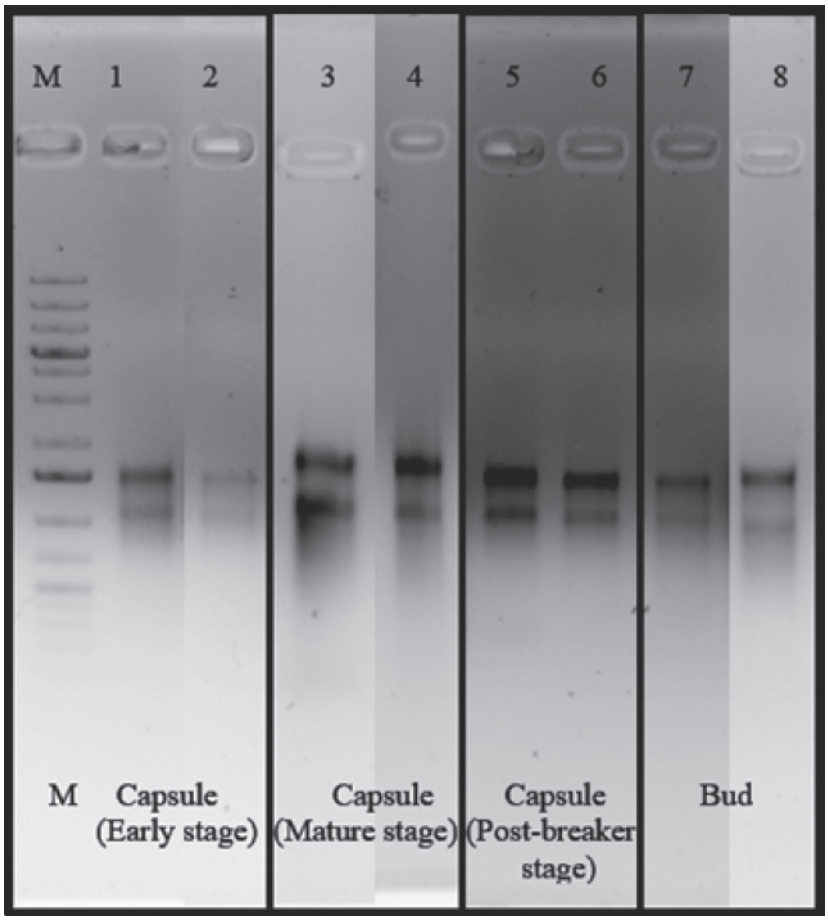

Fig. 3. Total RNAs isolated from flower bud and capsule tissues of *Impatiens balsamina* using modified CTAB method on 1% (w/v) agarose gel. M, Gene Ruler 10kb plus DNA ladder marker. Lane 1-2, total RNA from the capsules in early stage; Lane 3-4, total RNA from the capsules in mature stage; Lane 5-6, total RNA from the capsules in post-breaker stage; Lane 7-8, total RNA from the flower buds. Volume of RNase-free water used to dissolve/elute total RNAs was the same in all total RNA extraction methods.

In conclusion, a RNA extraction method was developed to extract total RNA from *I. balsamina* by modifying the CTAB method (using high volumes of extraction buffer) in combination with spin columns (silica-membrane filtration) purification and incorporation of a DNase I treatment step. It is a relatively simple and fast method to obtain high quantity and quality of total

RNA for NGS (transcriptomics) application. This method might be useful for total RNA extraction of other medicinal herbaceous plants with high secondary metabolites such as *I. balsamina*.

ACKNOWLEDGEMENT

This research was financially supported by Fundamental Research Grant Scheme (FRGS/2/2014/SG05/TAYLOR/02/1) from Ministry of Higher Education.

REFERENCES

Baskar, N., Devi, B.P. & Jayakar, B. 2012. Anti-cancer studies on ethanol extract of impatiens balsamina. *International Journal of Research in Ayurveda & Pharmacy,* **3(4)**: 631-633.

Bohm, B. & Towers, G. 1962. A study of phenolic compounds in Impatiens. *Canadian Journal of Botany,* **40(5)**: 677-683.

Carra, A., Gambino, G. & Schubert, A. 2007. A cetyltrimethylammonium bromide-based method to extract low-molecular-weight RNA from polysaccharide-rich plant tissues. *Analytical Biochemistry,* **360(2)**: 318-320.

Chang, S., Puryear, J. & Cairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter,* **11(2)**: 113-116.

Chomczynski, P. & Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry,* **162(1)**: 156-159.

Christou, A., Manganaris, G.A., Papadopoulos, I. & Fotopoulos, V. 2013. Hydrogen sulfide induces systemic tolerance to salinity and non-ionic osmotic stress in strawberry plants through modification of reactive species biosynthesis and transcriptional regulation of multiple defence pathways. *Journal of Experimental Botany,* **64(7)**: 1953-1966.

Dictionary of Chinese Crude Drugs. 2003. Shang Hai Scientific Technological Publishers, Shanghai, PR China.

Ding, Z.-S., Jiang, F.-S., Chen, N.-P., Lv, G.-Y. & Zhu, C.-G. 2008. Isolation and identification of an anti-tumor component from leaves of *Impatiens balsamina*. *Molecules,* **13(2)**: 220-229.

Ekblom, R. & Galindo, J. 2011. Applications of next generation sequencing in molecular ecology of non-model organisms. *Heredity,* **107(1)**: 1-15.

Gehrig, H.H., Winter, K., Cushman, J., Borland, A. & Taybi, T. 2000. An improved RNA isolation method for succulent plant species rich in polyphenols and polysaccharides. *Plant Molecular Biology Reporter,* **18(4)**: 369-376.

Hargen Jr, C. 1966. The differentiation of pigment of flower parts. 1. Flavonoid pigments of Impatiens balsamina, genotype 11HP'P', and their distribution within the plant. *American Journal of Botany,* **53**: 46-54.

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 Methods,* **10(1)**: 1.

Iandolino, A.B., Goes da Silva, F., Lim, H., Choi, H., Williams, L.E. & Cook, D.R. 2004. High-quality RNA, cDNA, and derived EST libraries from grapevine (*Vitis vinifera* L.). *Plant Molecular Biology Reporter,* **22(3)**: 269-278.

Ishiguro, K., Ohira, Y. & Oku, H. 1998. Antipruritic Dinaphthofuran-7,12-dione Derivatives from the Pericarp of *Impatiens balsamina*. *Journal of Natural Products,* **61(9)**: 1126-1129.

Jaakola, L., Pirttila, A.M., Halonen, M. & Hohtola, A. 2001. Isolation of high quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. *Molecular Biotechnology,* **19(2)**: 201-203.

Kang, S.-N., Goo, Y.-M., Yang, M.-R., Ibrahim, R.I.H., Cho, J.-H., Kim, I.-S. & Lee, O.-H. 2013. Antioxidant and antimicrobial activities of ethanol extract from the stem and leaf of *Impatiens balsamina* L. (Balsaminaceae) at different harvest times. *Molecules,* **18(6)**: 6356-6365.

Kansal, R., Kuhar, K., Verma, I., Gupta, R.N., Gupta, V.K. & Koundal, K.R. 2008. Improved and convenient method of RNA isolation from polyphenols and polysaccharide rich plant tissues. *Indian Journal of Experimental Biology,* **46(12)**: 842-845.

Liew, K., Yong, P.V.C., Navaratnam, V., Lim, Y.M. & Ho, A.S.H. 2015. Differential proteomic analysis on the effects of 2-methoxy-1, 4-naphthoquinone towards MDA-MB-231 cell line. *Phytomedicine,* **22(5)**: 517-527.

Lim, Y.-H., Kim, I.-H., Seo, J.-J. & Kim, J.-K. 2006. Tyrosinase inhibitor from the flowers of *Impatiens balsamina*. *Journal of Microbiology and Biotechnology,* **16(12)**: 1977-1983.

Little, J.E., Sproston, T.J. & Foote, M.W. 1948. Isolation and antifungal action of naturally occurring 2-methoxy-1, 4-naphthoquinone. *Journal of Biological Chemistry,* **174(1)**: 335-342.

Na, D., Hefen, L. & Xiuren, Z. 2013. Comparation of different methods for RNA extraction from bud of *Impatiens balsamina*. *Journal of Henas Institute of Science & Technology,* **41(2)**: 20-23.

Nurul, A., Nur Arina, H., Bhore, S. & Shah, F. 2010. Total phenolic content and RAPD analysis of Garden Balsam (*Impatiens balsamina* L.) accessions from Malaysia. *Middle East Journal of Scientific Research,* **5(6)**: 454-463.

Oku, H. & Ishiguro, K. 2001. Antipruritic and antidermatitic effect of extract and compounds of *Impatiens balsamina* L. in atopic dermatitis model NC mice. *Phytotherapy Research,* **15(6)**: 506-510.

Oku, H. & Ishiguro, K. 2002. Cyclooxygenase-2 inhibitory 1, 4-naphthoquinones from *Impatiens balsamina* L. *Biological and Pharmaceutical Bulletin,* **25(5)**: 658-660.

Ong, J.Y.H., Yong, P.V.C., Lim, Y.M. & Ho, A.S.H. 2015. 2-Methoxy-1, 4-naphthoquinone (MNQ) induces apoptosis of A549 lung adenocarcinoma cells via oxidation-triggered JNK and p38 MAPK signaling pathways. *Life Sciences,* **135**: 158-164.

Porebski, S., Bailey, L.G. & Baum, B.R. 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reporter,* **15(1)**: 8-15.

Romero, I.G., Pai, A.A., Tung, J. & Gilad, Y. 2014. RNA-seq: impact of RNA degradation on transcript quantification. *BMC Biology,* **12(1)**: 1.

Sahu, S.K., Thangaraj, M. & Kathiresan, K. 2012. DNA extraction protocol for plants with high levels of secondary metabolites and polysaccharides without using liquid nitrogen and phenol. *ISRN Molecular Biology,* **2012**: 1-6.

Salzman, R.A., Fujita, T., Zhu-Salzman, K., Hasegawa, P.M. & Bressan, R.A. 1999. An Improved RNA Isolation Method for Plant Tissues Containing High Levels of Phenolic Compounds or Carbohydrates. *Plant Molecular Biology Reporter,* **17(1)**: 11-17.

Shoji, N., Umeyama, A., Saitou, N., Yoshikawa, K., Kan, Y. & Arihara, S. 1994. Hosenkosides A, B, C, D, and E, novel baccharane glycosides from the seeds of *Impatiens balsamina*. *Tetrahedron,* **50(17)**: 4973-4986.

Thevissen, K., François, I.E., Sijtsma, L., van Amerongen, A., Schaaper, W.M., Meloen, R., Posthuma-Trumpie, T., Broekaert, W.F. & Cammue, B.P. 2005. Antifungal activity of synthetic peptides derived from *Impatiens balsamina* antimicrobial peptides Ib-AMP1 and Ib-AMP4. *Peptides,* **26(7)**: 1113-1119.

Vasanthaiah, H.K., Katam, R. & Sheikh, M.B. 2008. Efficient protocol for isolation of functional RNA from different grape tissue rich in polyphenols and polysaccharides for gene expression studies. *Electronic Journal of Biotechnology,* **11(3)**: 42-51.

Wang, Y.-C., Li, W.-Y., Wu, D.-C., Wang, J.-J., Wu, C.-H., Liao, J.-J. & Lin, C.-K. 2011. *In Vitro* Activity of 2-methoxy-1, 4-naphthoquinone and Stigmasta-7, 22-diene-3β-ol from *Impatiens balsamina* L. against Multiple Antibiotic-Resistant *Helicobacter pylori*. *Evidence-Based Complementary and Alternative Medicine,* **2011**: 1-5

White, E.J., Venter, M., Hiten, N.F. & Burger, J.T. 2008. Modified cetyltrimethylammonium bromide method improves robustness and versatility: the benchmark for plant RNA extraction. *Biotechnoly Journal,* **3(11)**: 1424-1428.