Description of New Methods

Optimization of RNA extraction protocol for *Mentha piperita*

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Abstract

With regard to the high levels of secondary metabolites in peppermint (*Mentha piperita*) leaves, we evaluated two different methods for total RNA isolation of this medicinal plant to find the best extraction method, which provides high quality RNA. The methods under investigation included RNX-plus and modified RNX-plus (1-5). The visualization of clear 28S RNA and 18S RNA bands on 1% agarose gels was used to check RNA quality. In the modified method (RNX-plus), the purity of 28S and 18S rRNA was highly acceptable when assessed in an agarose denaturing gel. Following the superior quality and quantity of RNA isolated in this method, it is recommended for RNA isolation from *Mentha piperita*. The final analysis to get the best RNA isolation method was gene expression analysis using tubulin primer as an internal gene. The obtained results demonstrated that it is possible to extract total RNA of reasonable quality and quality from *Mentha piperita* rich in polysaccharides and polyphenols by using the modified RNX protocol number 2.

Keywords: medicinal plants, *Mentha piperita***,** polyphenol, polysaccharide, RNA isolation

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Introduction

A valuable family of plants is Lamiaceae, which has been used in traditional medicine for centuries. A Lamiaceae genus, *Mentha* includes different species that are distributed worldwide, especially in temperate regions of Australia, South Africa, and Eurasia (Dorman et al., 2003; Krzyzanowska et al., 2011). High RNA quality and integrity in plants are required for molecular investigation (Gehrig et al., 2000; Kiefer et al., 2000). On the other hand, molecular study with aromatic plants is very difficult because of the high content of polyphenolic and polysaccharide compounds (Poyraz et al., 2010). The critical step for a successful RT-PCR is the extraction of high quality RNA from plant tissues. Components such as secondary metabolites, phenolics, and polysaccharide compounds act as inhibitors and can cause inaccurate and undesirable results (Canpaylan et al., 2014). Therefore, extracting high-quality nucleic acids from plant tissues abundant in polysaccharides and polyphenols is usually a complicated procedure. The quality and quantity of the isolated nucleic acids are affected by the presence of these substances (Heidari Japelaghi et al., 2011). Also, these compounds inhibited DNA polymerase, DNA restriction endonuclease, and RNA-dependent reverse transcriptase activities (Iandolino et al., 2004; Moser et al., 2004). Many studies have shown that *Mentha* plants are rich in phenolic compounds,

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Fig I. Preparation and sampling peppermint (*Mentha piperita*) plants

particularly phenolic acids and flavonoids (Pereira and Cadoso, 2013).

The amounts of total phenolic compounds (such as gallic acid) and flavonoid (such as quercetin) in leaves of *Mentha piperita* were 360.04±0.285 and 421.96±0.25 mg/100gm, respectively (Dyab et al., 2015). HPLC analysis showed that *Mentha piperita* mainly comprised of polysaccharides like arabinose, galacturonic acid, galactose, glucose, and glucuronic acid (Liu et al., 2014). The SDS-LiCl and Modified CTAB methods were used for RNA extraction in *Fritillaria unibracteata* and the low purity of RNA was observed (Hou et al., 2011). Result of RNA isolation using TRIzol kit showed that it is not an effective method in many medicinal plants (Deepa et al., 2014; Liu et al., 2018). Employing RNeasy Plant Mini Kit in removing polysaccharides had a weak performance and led to degradation of RNA (Liu et al., 2018). Regarding the high phenolic and polysaccharide content in *Mentha piperita*, we presented an optimized method for RNA extraction based on different methods.

Material and Methods

Plant material and RNA extraction

The rhizomes of peppermint plants were supplied by the Iranian Institute of Medicinal Plants, Karaj, Iran. Rhizome fragments (10-15 cm long) were planted in pots and watered and fertilized twice a month (Fig. I). The growth conditions were 25/18 ℃ (day/night) with a light density of 35000-40000 lx. Samples were collected at the flowering stage before they were immediately frozen in liquid nitrogen and stored at -80 ℃. We evaluated two RNA extraction protocols, namely RNX-plus (Cinagene) and the RNX-plus modified (1-5) methods using the leaf tissues of *Mentha piperita.*

Method 1: RNA was extracted by RNX-plus according to the Cinagene protocol:

1. One (1) ml of the ice-cold RNX–plus solution was added to a 2 ml tube containing a homogenized sample.

2. A vortex was applied for 5-10 seconds, followed by keeping the solution at room temperature5 minutes.

3. In the third step, 200 µl of Chloroform was added.

4. Shaking for mixing well was used for 15 seconds (without vortex).

5. The mixture was incubated on the ice, at 4 ℃ for 5 minutes.

6. Centrifugation was conducted at 12000 rpm, under 4 ℃, and for 15 minutes.

7. The aqueous phase was transferred to a new RNase-free 1.5 ml tube, and then, an equal amount of isopropanol was added.

8. The mixture was blended slowly and incubated on the ice for 15 minutes.

9. The mixture was centrifuged at 12000 rpm at 4 ℃ for 15 minutes.

10.The supernatant was removed by centrifugation, and 1 ml of ethanol (75%) was added; a brief vortex was used to re-suspend the pellet, and it was centrifuged at 4 ℃ for 8 minutes at 7500 rpm.

11.The supernatant was discarded by centrifuging, and the pellet was left at room temperature to dry for a few minutes.

12.The pellet was dissolved in 50 µl of DEPC-treated water. To help it dissolve, the tube was put in a water bath (55-60 ℃) for 10 minutes.

(Fermentas, Canada), and oligo (dT)18 in a total of 20 µl reaction mixture, according to the manufacturer's instructions. In this study, the reference gene was tubulin (Table 1). PCR was performed by using samples, primers (5 pmol), and Master Mix (CinnaGen, Iran). For amplifications, the following program was used: 94 ℃ for 2 min, followed by 30 cycles of 94 ℃ for 45 s, (annealing temperature for each gene) for 45 s, and 72 ℃ for 45 s.

Table 1
Sequence, annealing temperature, and product size of the tubulin primers used in RT-PCR

Gene	Primer	Sequence (5'-3')	Annealing Temperature (°C)	Product Size (bp)
tub	Forward	GACCACCTCATGAAGATCTTAACC	57	680
	Reverse	ATTCTCGTAGTCCAAAGC		

Modified RNX-plus

1. Applied changes: keep in RNX solution for 1 hour, wash with chloroform three times, and keep in isopropanol for 1 hour.

2. Applied changes: keep in RNX solution for 5 h, wash with chloroform twice, and keep in isopropanol for 1 h.

3. Applied changes: keep in RNX solution for 4 h, wash with chloroform three times, and keep in isopropanol for 2 h.

4**.** Applied changes: keep in RNX solution overnight for 13 hours, wash twice with chloroform, once for 10 minutes on ice; then, for 5 minutes on ice, and keep in isopropanol for one hour.

5**.** Applied changes: keep in the RNX solution for three hours, wash with chloroform twice; first, incubate on ice for ten minutes; then, incubate on ice for five minutes, and stay at isopropanol for overnight (12 h).

cDNA synthesis and RT-PCR reaction

Reverse transcription reaction was performed for first-strand cDNA synthesis using 3-5 µg of purified total RNA, the RevertAid™ Reverse Transcriptase

Results

In this research, two methods were used, as mentioned in the previous section. The extracted total RNA samples were stored in - 80 ℃. The integrity of RNA was evaluated by visualization of intact 28S and 18S rRNA bands on 1% agarose gels (Fig. II). For all RNA samples in this study, two bands, namely 28S and 18S rRNA, were detected on agarose gels. However, the result of gel electrophoresis for the RNX-plus method showed that the bands of RNA were not clear enough, which demonstrates that the purity of RNA is not good (Fig. II. A). In the changed RNX-plus method, the 28S and 18S rRNA bands were visible when assayed in agarose gel. The 28S rRNA band became visible and was equal to or more abundant than the 18S rRNA band thereby, indicating that RNA degradation during extraction is minimal or has not occurred (Fig. II. B lane 2).

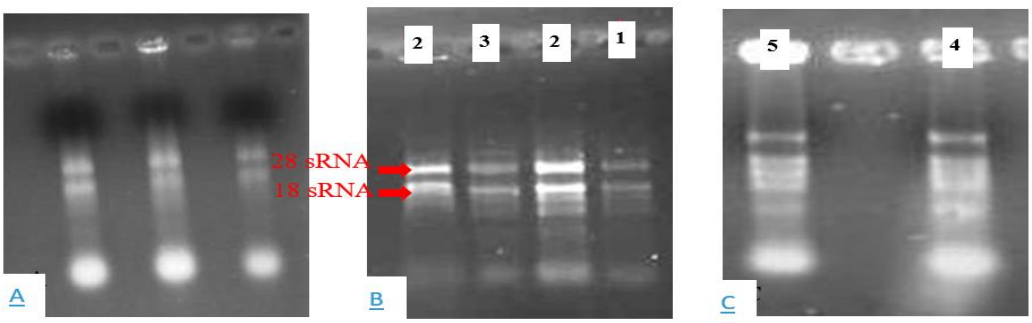

Fig II. Agarose gel electrophoresis of total RNA from the RNX-plus method (A) and the modified RNX-plus method (B and C: 1-5) of *Mentha piperita*

Concerning agarose gel results, a high-quality and adequate amount of RNA for the subsequent experiments could not be obtained using the RNX-plus method (Fig. II. A). Therefore, the integrity of RNAs extracted with the RNX-plus method was significantly lower than the modified RNX-plus method (number 2). Moreover, no visible bands were observed around the loading wells, which indicates that the RNA was free of genomic DNA contamination. In the modified methods (numbers 4 and 5) around the loading wells, a band was observed, which shows the RNA is contaminated by genomic DNA (Fig II. C). Visible ribosomal RNA bands, including 28S and 18S rRNA without smearing, demonstrated the purity of isolated RNA in this experiment (Fig. II. B). Accordingly, the RNA sample isolated with modified RNX-plus number 2 was not degraded and was free of genomic DNA.

In this experiment, we used the tubulin primer as a reference gene. Results showed no PCR amplification of the tubulin gene when cDNA fragment synthesis was performed from RNA isolated with RNX-plus methods and Modified RNX-plus methods 1, 3, 4, and 5 (Fig. III. Lanes 2, 3, 5, and 7). At the same time, a distinct tubulin-band was obtained in cases when cDNA fragment synthesis from RNA isolated with modified RNX-plus method 2 was performed (Fig. IV. Lane 2). The expected tubulin gene band of 680 bp in size was obtained on agarose gel. The improved RNX-plus method number 2 showed the high quality of the isolated RNAs that can be used in other molecular processes.

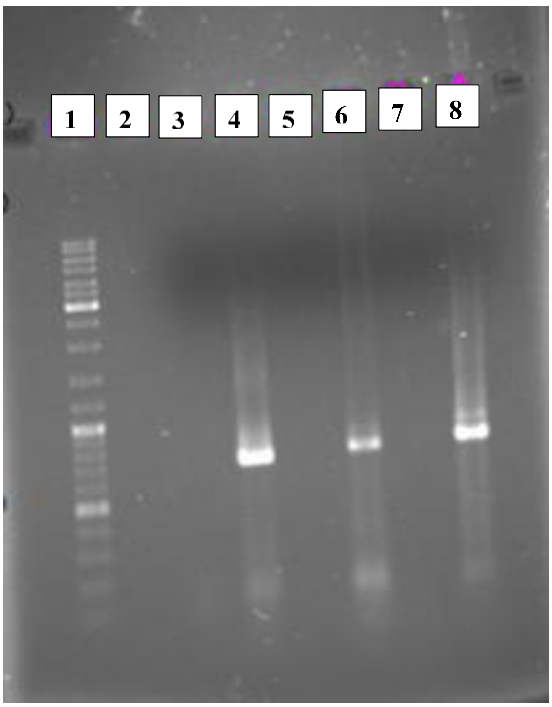

Fig. III. Agarose gel electrophoresis of RT-PCR products obtained from leaf total RNA for tubulin; 1: DNA ladder (1 kb Plus); 2: modified RNX-plus number 1; 3: modified RNX-plus number 3; 4- 6- 8: control $^{+}$; 5: modified RNX-plus number 4; 7: modified RNX-plus number 5

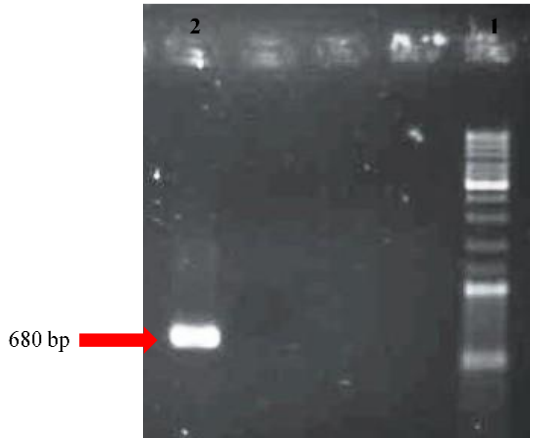

Fig. IV. Agarose gel electrophoresis of RT-PCR products obtained from leaf total RNA for tubulin (lane 1: DNA ladder 1 kb Plus, lane 2: modified RNX-plus number 2).

Discussion

Today, transgenic plants are used in many studies, including those on medicinal and aromatic plants. Moreover, recombinant DNA technology is applied to change the essential oil composition. Therefore, isolating RNA with high quality and purity is the first step in achieving these goals (Poyraz et al., 2009). In RT-PCR applications, the extraction of total RNA is necessary. However, RNA isolation from aromatic plants might be difficult because of the presence of a high accumulation of polyphenols, polysaccharides, and other secondary metabolites (Wang et al., 2005; Provost et al., 2007). Nevertheless, there are still some problems associated with total RNA isolation methods. For example, several methods were used for RNA isolation in medicinal plants, but they had problems such as RNA degradation, poor quality, and low purity (Hou et al., 2011; Deepa et al., 2014; Liu et al., 2018). Therefore, different methods of RNA extraction were checked in this study to obtain high-quality RNA for molecular experiments. The last RNA quality

analysis was RT-PCR using cDNA fragments. The availability of the isolated RNAs was tested by using RNAs as a template to amplify the tubulin gene.

The carbohydrate and polyphenol contamination probably inhibited the reverse transcriptase enzyme's action for cDNA synthesis. RNX-plus buffer is a combination of phenol, SDS, and EDTA. It has been shown by previous researchers that phenol seems to be a strong protein denaturant and RNase inhibitor; also, SDS and EDTA are suitable RNase inhibitors (Chomczynski and Sacchi, 2006).
Moreover, using isopropanol caused efficient precipitation of RNA from the contaminating proteins and polysaccharides. Autoclaved DEPC-treated water produced enough of an aqueous environment for the RNA to partition into the aqueous phase (George, 2018). Other researchers indicated that chloroform effectively removes proteins (Asif et al., 2006; Chan et al., 2007; Ma et al., 2015). In conclusion, our results indicated the isolation of intact RNA with high yield and quality from leaves of *Mentha piperita* plants by using the modified RNX-plus protocol number 2. This protocol can be used for other aromatic plant tissues with high concentrations of phenolic and polysaccharide compounds for the next RT-PCR and molecular studies.

In sum, the modified RNX-plus protocol number 2 assisted in obtaining intact, high-yield, and quality RNA from the leaves of *Mentha piperita* plants. Although this protocol does not require specialized equipment and is efficient, less expensive, and simple, new optimized protocols must be developed for different plant species and tissues.

References

Asif, M., P. Trivedi, T. Solomos and M, Tucker. 2006. Isolation of high-quality RNA from apple (*Malus domestica*) fruit. Journal of Agricultural and Food Chemistry, 54: 5227–5229.

Canpaylan, I., A. Candar, M. Gumus, S. Pazarlar and S. Onder. 2014. A comparison of six total RNA isolation methods for diagnosis of gysvd-1 (Grapevine yellow speckie viroid-1) on *vitis vinifera* L. leaves. Pakistan Journal of Botany, 46 (6): 2089-2093.

Chan, K.L., C.L. Ho, P. Namasivayam and S. Napis. 2007. A simple and rapid method for RNA isolation from plant tissues with high phenolic compounds and polysaccharides. Protocol Exchange, 184:1-6.

Chomczynski, P. and N. Sacchi. 2006. The single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction : twenty-something years on. Nature Protocols, 1(2): 581–585.

Deepa, K., Sheeja, T.E., Santhi, R., Sasikumar, B., Cyriac, A., Deepesh, P.V., Prasath, D. 2014. A simple and efficient protocol for isolation of high quality functional RNA from different tissues of *Curcuma longa*. Physiology and Molecular Biology of Plants, 20 : 263-271.

Dorman, H. J. D.; M. Kosar, K. Kahlos, Y. Holm and R. Hiltunen. 2003. Antioxidant properties and composition of aqueous extracts from Mentha species, hybrids, varieties and cultivars. Journal of Agricultural and Food Chemistry, 51(16): 4563-4569.

Dyab, A.S., A.M. Aly and H.I. Matuk. 2015. Enhancement and evaluation of Peppermint (*Mentha piperita* L.) beverage. International Journal of Life Sciences Research, 3 (1): 175-185.

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

George, A. 2018. Simple and efficient method for functional RNA extraction from tropical medicinal plants rich in secondary metabolites. Tropical Plant Research, 5 (1): 8-13.

Heidari Japelaghi, R., R. Haddad and Gh.A. Garoosi. 2011. Rapid and efficient isolation of high quality nucleic acids from plant tissues rich in polyphenols and polysaccharides. Molecular Biotechnology, 49:129-137.

Hou, P., Xie, Z., Zhang, Le., Song, Z., Mi, J., He, Y. and Li, Y. 2011. Comparision of three different methods for total RNA extraction from *Fritillaria unibracteata*: a rare Chinese medicinal plant. Journal of Medicinal Plants Research, 5 (13): 2834-2838.

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

Kiefer, E., W. Heller and D. Ernst. 2000. A simple and efficient protocol for isolation of functional RNA from plant tissues rich in secondary metabolites. Plant Molecular Biology Reporter, 18: 33–39.

Krzyzanowska, J.; B. Janda, L. Pecio, A. Stochmal, W. Oleszek, A. Czubacka, M. Przybys and T. Doroszewska. 2011. Determination of polyphenols in *Mentha longifolia* and *M. piperita* field-grown and in vitro plant samples using UPLC-TQ-MS. Journal of AOAC International, 94(1): 43-50.

Liu, X., Z.L. Sun, A.R. Jia, Y.P. Shi, R.H. Li and P.M. Yang. 2014. Extraction, preliminary characterization and evaluation of in vitro antitumor and antioxidant activities of polysaccharides from *Mentha piperita*. International Journal of Molecular Science, 15 (9): 16302-16319.

Liu, L., Han, R., Yu, N., Zhang, W., Xing, L., Xie, D., Peng, D. 2018. A method for extracting high quality total RNA from plant rich in polysaccharides and polyphenols using dendrobium houshanese. Plos One, 13 (5): 1-9.

Ma, Z., B. Huanng, Sh. Xu, Yu. Chen, Sh. Li, Sh. and S. Lin. 2015. Isolation of high quality total RNA from chines Fir. Plos One, 10 (6): 2-10.

Moser, C., P. Gatto, M. Moser, M. Pindo and R. Velasco. 2004. Isolation of functional RNA from small amounts of different grape and apple tissues. Molecular Biotechnology, 26: 95–99.

Pereira, O.R. and S.M. Cardoso. 2013. Overview on Mentha and Thymus polyphenols. Current Analytica Chemistry, 9: 282-296.

Poyraz, I., Sozen, E. and Arslanyolu, M. 2010. Isolation of quality total RNA from the aromatic plant *Origanum onites*. Naturforsch, 65: 266-270.

Provost G. L., R. Herrera, J.A. Paiva, P. Chaumeil, F. Salin and C. Plomion. 2007. A micromethod for high throughput RNA extraction in forest trees. Biological Research, 40: 291–297.

Wang, T., N. Zhang and L. Du. 2005. Isolation of RNA of high quality and yield from *Ginkgo biloba* leaves. Biotechnology Letters, 27: 629–633.