Journal of Nuts

Journal homepage: sanad.iau.ir/journal/ijnrs/

ORIGINAL ARTICLE

Isolation and Purification of High-quality RNA from Pistachio (*Pistacia vera* L.)

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K E Y W O R D S

Isolation;
Mini kit;
Pistachio;
Real time;
RNA;
TRIzol

A B S T R A C T

Pistachios (*Pistacia vera* L.) are known as one of the nuts with high nutritional value, and an important commercial product worldwide. Pistachio is a tree that is compatible with dry climate regions and has the largest cultivated area in Iran. Pistachio yields have declined in recent years due to the rise in soil salinity caused by climate change (Mohit Rabari *et al.*, 2023; Behzadi Rad *et al.*, 2021). Nevertheless, the presence of phenolic compounds and other secondary metabolites may impede the isolation of high-quality RNA from pistachio leaves in numerous instances. In the present study, several methods were used to purify the total RNA of Shahpasand variety. These methods included Trizol kit; mini kit and modified method (TRIzol & mini kit) were used. The obtained results showed the superiority of modified method (TRIzol & kit) compared to TRIzol and Kit methods. In this method, the A260/A280 ratio of RNA sample was 2.12. In the next step, cDNA was synthesized and used to analyze SOS1 gene expression via Real Time PCR. Gene-specific amplification was confirmed by a single peak in the melt-curve analysis. Furthermore, Ct values showed that SOS_1 gene extracted by the (TRIZOLE & Kit) was the most abundant transcript with a mean Ct value of 20 whereas in two other ways was the least abundantly transcribed. In conclusion, the results of this investigation demonstrated that the optimized extraction method is capable of isolating high-quality RNA from pistachios. The extracted RNA can be used for further molecular studies, such as real-time PCR and other downstream applications.

Introduction

Pistachio (*Pistacia vera* L.), known as green gold, is a member of the Anacardiaceae family. Its only economically important species is *P. vera* (Nazoori *et al*., 2022; Moazzam jazi *et al*., 2017). This tree originated from Central Asia, and then was cultivated throughout the Middle East and the Mediterranean region (Hosseini *et al*., 2022; Nazouri *et al*., 2022). The history of this plant goes back to 3000-4000 years ago in Iran, which is compatible with the geographical conditions of Iran (Norouzi *et al*., 2018). Since then, the cultivated area of this product has increased about 10,000 to 12,000 hectares per year. (Babaei *et al*., 2021). Iran, America, Turkey, and Syria are now the world's major pistachio producers, with Iran leading the pack with an average of 298,838.67 tons (Faostat, 2016). In addition to its commercial usefulness, the pistachio tree is nutritionally and medicinally important. Various parts of the plant, including flowers, leaves, seeds, and resin derived from the stem, have antioxidant and antimicrobial activities (Roozban *et al*., 2006; Nazoori *et al*., 2024; Sharifkhah *et al*., 2020; Fathalizadeh *et al*., 2015). In recent years, the agricultural industry has made

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Received: 9 January 2024; Received in revised form: 10 February 2024; Accepted: 5 March 2024
DOI: 10.60680/jon.2024.4011261

significant progress, while pistachio cultivation has not changed in terms of different weather conditions.

On the other hand, the high level of secondary metabolites has made RNA purification difficult. Quality RNA extraction is essential for molecular processes, such as genomic libraries (Seçgin *et al*., 2020; Chang *et al*., 2016). The content of secondary metabolites increases in plants that are subjected to biotic and abiotic stresses (Moazzam jazi *et al*., 2015). In the extraction process, polyphenol compounds and polysaccharides have the greatest effect (Honaas *et al*., 2017). Phenolics are readily oxidized and covalently linked to quinones; the created compound has a high tendency to bind to proteins and nucleic acids and causes the insolubility of high molecular weight complexes. One of the inhibitors of RNA extraction are polysaccharides, which tend to precipitate with RNA if the ionic strength of extraction solution is low. (Majidi and Bahmani, 2018). To decrease the effect of polyphenols and secondary metabolites in RNA extraction, many procedures are used, including extraction kits, trizol, CTAB buffer, and cesium chloride. We describe an optimization method for RNA extraction from a Pistachio that can be applied for molecular studies, such as gene expression by real-time PCR. Our intent in presenting this modified method is not to oppose other methods that work well for specific crops.

Materials and Methods

Young leaves of 20-year-old Shahpas and cultivar trees were collected from the farm of Damghan Pistachio Research Center in May. To isolate RNA, the workbench was washed with 70% ethanol. The sampler heads were autoclaved to remove contamination.

TRIzol protocol

0.1 to 0.3 gr of young leaf tissue was taken and powdered by helping liquid nitrogen. In the next step, RNA extraction was performed using the TRIzol kit (yektatajhiz).

Total RNA extraction mini kit

In the first step, the leaf sample was weighed at the rate of 30 mg. The weighed leaves were homogenize using liquid nitrogen and transferred to a new microtube. 350 microliters of FARB buffer and 3.5 microliters of 2-ME were used. The sample mixture was transferred to the filter column and centrifuged. The clarified supernatant was added to a new microtube, and 1.5 volumes of 70% ethanol without RNase. The desired sample was transferred to FARB Mini column. Centrifuge at 14500 for 1 minute and the supernatant was removed.

250 µL of Wash Buffer 1 was poured into the mini column by the sampler and centrifuged. The supernatant solution was removed and this step was repeated again. Add 750 µL of Wash Buffer 2 to the mini FARB column, centrifuge at 14000 rpm for 1 min and discard the supernatant. To dry the mini-FARB column, we centrifuged it at full speed to dry it. FARB mini-column was placed in a 1.5 microtube and 40 microliters of RNase-free ddH2O was added to the center of the FARB membrane. Centrifuge the mini-FARB column at full speed for 1 min to elute the RNA. RNA was stored at -70°C for subsequent steps.

Optimize RNA extraction (kit and TRIzol)

In this method, YTzol solution and column extraction kit were used with modifications. In the first step, plant tissue (frozen at -70) was powdered using liquid nitrogen and ground with a mortar. Homogenize tissue samples in 1 ml YTzol and 5 Microliters of ß-mercaptoethanol were added per 100 mg tissue using a tissue homogenizer or rotor-stator. The nucleoprotein complex was completely lysed after the sample was incubated at 30°C for 15 minutes. The supernatant was transferred to a new microtube without Rnase following the centrifugation of the sample. This step can remove protein, fat, polysaccharide, muscle or fiber. 200 microliters of

chloroform were added to the new microtube; the sample was inverted 30 times and rested on dry ice for 20 minutes. Then, the samples were centrifuged and the clear phase was transferred to a microtube containing a FARB Mini column and 2.3 volumes of isopropanol were added to it. The sample was centrifuged at 14000 rpm for 60 seconds, and the columnar microtube was transferred to the Collection Tube. Add 700 µL of Wash Buffer 1 to the mini FARB column and centrifuge at 14000 rpm for 60 seconds. The upper aqueous phase was removed, and the columnar microtube was transferred to the Collection Tube. Add 1000 µL of Wash Buffer 2 to mini-FARB column and centrifuge at 14000 rpm for 60 seconds. The supernatant was removed and this step was repeated two more times. To dry the columnar microtube, it was centrifuged, then transferred to a new microtube and 60 microliters of DEPCE water was added to it.

Quantification and qualification of isolated RNA

The quantity of extracted RNA was measured using NanoDrop spectrophotometer. For this purpose, the device with using 100 microliters of distilled water (RNA solvent) Calibrated. After, 98 microliters of sterile distilled water and 2 microliters RNA. It was poured into the cuvette and mixed slowly with the sampler (must be careful that the RNA does not break), and in wavelength 260/280 nm was tested and optical density (OD) was determined. Maximum optical absorption Nucleic acids were detected at a wavelength of 260 nm. If the optical absorption ratio 260A/280A is in range of 1.8-2, it indicates the high purity of the nucleic acid and it can be used in future works. The quality of extracted RNA was confirmed by the horizontal electrophoresis.

cDNA Synthesis

cDNA synthesis was performed using a synthesis kit (yektatajhiz) with Oligo (dT) primer. Finally, the synthesized samples were placed in a freezer.

Real-Time PCR Analysis

The resulting cDNA was used as template for the amplification of SOS_1 gene using specific primers and real-time PCR technology (Yektatajhiz, Iran), with GAPDH used as a reference. Primers were designed using the sequence of studied genes in the NCBI database and using primer3 and oligo7 software (Table 1).

Table 1. Sequence and properties of designed primers for the reaction.

Primer name	sequences (5'-3')	Gen Bank accession number	Tm (°C)	PCR product length (bp)
SOS1-forward	CGACGATATCCCCAGGGCTT	XM_031407501.1	60	97
SOS1-revers	GTTGGCGCTCTTGACAGACG		62.5	

To ensure the non-replication of genomic DNA, all primer pairs were designed as exon-exon junctions. Real-time PCR reactions were done with Syber green kit (Yektatajhiz, Iran). Finally, an agarose gel electrophoresis of the real-time PCR reaction products was performed to guarantee the precision of the amplification statistical analysis. Quantitative Real-Time PCR data were analyzed using the threshold cycle (Ct) method described by Livak *et al* (2001). Data were analyzed through SPSS software ver. 26 and GraphPad Prism 9.

Results

In order to purify RNA with high quality, three methods Trizol methods, RNA extraction kit and combined method were used. The OD 260/280 obtained from the obtained RNAs is shown in Table 2. The results of this study showed many varieties among the methods of RNA extraction in terms of quantity and quality. The obtained results indicate that the extracted RNA by helping Trizol and the yekta tajhiz kit was not of good quality (Table 2). Furthermore, the bands created on the agarose gel were not clear (Fig. 1a, b). However, the amount of

RNA produced by the combined method (Trizol and kit) has been significant (Figure 1c). Also, the absorbance of 260/280 was suitable. The amount of absorption obtained indicates proteins contamination (Table 2). The bands created on the agarose gel were clear and the 28S and 16S ribosomal regions were distinguishable. (Fig. 1c). In next step, gene expression of SOS1 was tested.

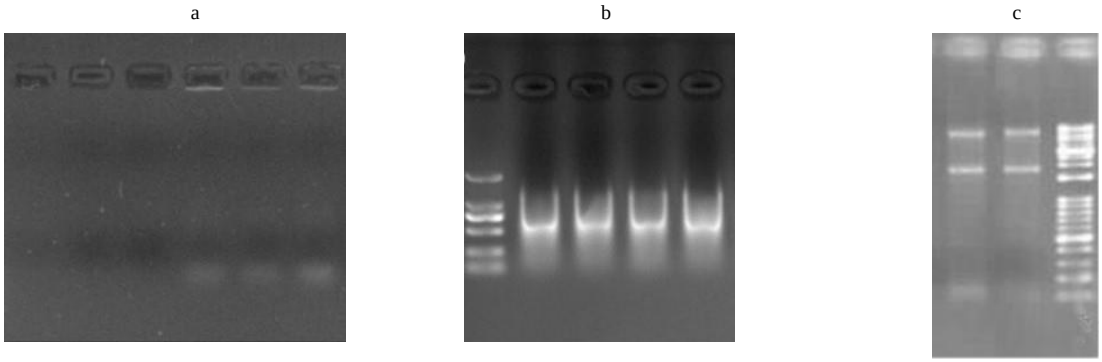

Fig. 1. a) RNA extraction with, b) Trizol method, mini kit, c) optimize kit & trizol.

Table 2. Yield of total RNA isolation

RNA isolation method	organ	A260/A280[a]	A260/A230[a]	Yield (µg mg^{-1})[a]
TRIzol	leaf	1.4	0.31	97.4
Total RNA extraction mini kit	leaf	1.7	1.3	147.4
(Kit and TRIzol)	leaf	2.09	2.12	458

As shown in Fig. 2, the expected amplicon SOS gene are 97 bp. Real-time SOS gene was correctly performed, and the melting curve showed a single peak (Fig. 3c). Therefore, the Ct values showed that SOS_1 gene extracted by the (Trizole & Kit) was the most abundant transcript with a mean Ct value of 20 whereas in two other ways was the least abundantly transcribed (Table 3).

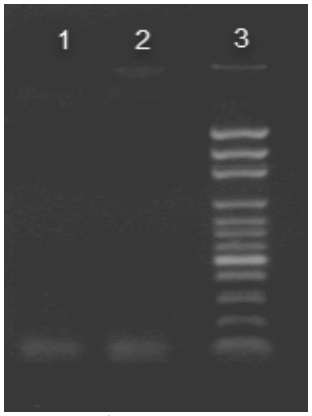

Fig. 2. Polymerase chain reaction products to verify the accuracy and precision of the primers SOS1 (97 bp)

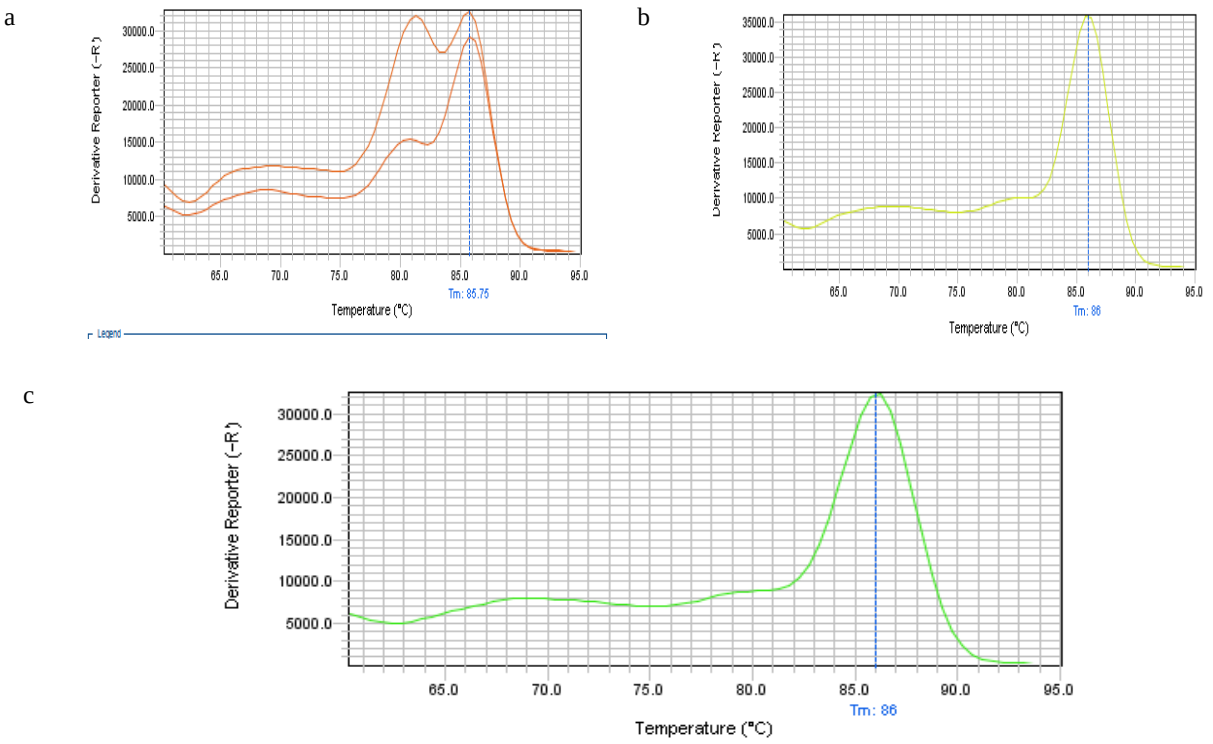

Fig. 3. The melting curve obtained from the amplification of the SOS gene extracted by the a) Trizol method, b) mini kit, c) optimize (Trizol & kit).

Table 3. The average ct real time PCR SOS1 gene by different methods.

	Method	Average of Ct
1	trizol	28.1
2	Mini kit	26.4
3	Trizol & kit	20

Discussion

The isolation of high-purity RNA is the first step in gene expression, regulation, and function (Xiao *et al*., 2012). Different methods of RNA isolation from pistachio tree were investigated. Optimization of high-purity RNA extraction is necessary for further applications such as real-time. In this report, we present a method that is based on the combination of Trizol and Kit, with modifications to the working method. This method does not necessitate numerous solutions, multi-step procedures, or multiple clean-ups to achieve satisfactory results. The two methods used for RNA extraction (mini kit and Trizol) were not suitable for pistachio plants, and the extracted RNA was of low quality (Table 1). While the total RNA extracted using the combined protocol (kit and TRIzol) has high quality and specific bands and can be used as an efficient method for pistachio extraction. (Fig. 1c). High secondary metabolites affect the purity of the extracted RNA and lower its quality (Amaranatha *et al*., 2020; Nadiya *et al.*, 2015). Such problems may be overcome by making some modifications to commercial kits and reagents. Using these methods is simple, fast, non-toxic and useful, which leads to the extraction of high-quality RNA (Liao *et al*., 2023; Tang *et al*., 2019; Chang *et al*., 2016). The guanidine thiocyanate–phenol solution, which is commercially available as TRIzol Reagent, disrupts the cells, denatures the proteins, and deactivates the nucleases, thereby stabilizing RNA (Honeywooda *et al*., 2022). The kenaf with high concentrations of polysaccharides and polyphenols readily undergoes oxidation. In this study, mercaptoethanol was used to reduce oxidative damage (Liao *et al*., 2023). On the other hand, in combination with the strong, but temporary denaturing effects of guanidinium isothiocyanate, any RNases present in

the sample will be completely inactivated (Yee *et al*., 2018). The extraction procedure was modified to incorporate chloroform, as the pistachio plant contains high levels of phenolic compounds. The biological phases are separated through the use of chloroform. After adding chloroform, 3 separate phases are created, the upper aqueous phase containing RNA, protein, and DNA is placed in the intermediate phase and organic (lower) phase (Zepeda *et al*., 2022; Toni *et al*., 2018). In the kit extraction method, 70% ethanol was used to precipitate RNA. While in the combined method, isopropanol was used. The obtained results showed higher accuracy and purity in this method. Due to its insolubility in ethanol and isopropanol, the addition of alcohol to the solution and subsequent centrifugation will cause the RNA to separate from the solution. If the RNA content in the sample is low, it has been suggested by Gangwar et al. (2021) and Arnold et al. (2013) that isopropanol may be more effective than ethanol in precipitating accessible proteins. The extracted RNA had purity and integrity and was suitable for downstream reactions (real-time PCR and RNA-seq). These applications were chosen because of their high sensitivity to impurities, and integrity of RNA (Tajner *et al*., 2013). Figures 2 show the resolved PCR products on 2 % agarose gel following amplification of SOS1 gene. Quality of extracted RNA was confirmed (Moazzam Jazi *et al*., 2015). Moreover, the Ct values showed that SOS1 gene extracted by the (Trizole & Kit) was the most abundant transcript with a mean Ct value of 20 whereas in two other ways was the least abundantly transcribed.

Conclusions

The optimized protocol in this paper is suitable for pistachio tree with high secondary metabolites. The purity and integrity of extracted RNA was confirmed using real time pcr. The method is preferable to other methods in that it excludes hazardous compounds, such as phenol, and the RNA samples are extracted with long-term stability. Moreover, in this method, amount of plant needed for extraction is small. The optimized protocol reported here may be useful for RNA isolation from leaves of pistachio and it is expected that the extracted RNA will be suitable for more RNA-related investigations, including gene expression, RNA-seq and downstream applications.

Acknowledgements

The authors would like to thank the Faculty of Agriculture of Azad Damghan University and Education Center for providing us with facilities to implement this project.

Conflict of interests

The authors declare that there is no conflict of interest.

References

Amaranatha RV, Somayanda I, Doherty CJ, Jagadish Sk (2020) A universal method for high-quality RNA extraction from plant tissues rich in starch, proteins and fiber. Scientific Reports. https://doi.org/10.1038/s41598-020-73958-5.

Arnold ME, Slomka MJ, Coward VJ, Mahmood S, Raleigh PJ, Brown IH (2013) Evaluation of the pooling of swabs for real-time PCR detection of low titre shedding of low pathogenicity avian influenza in turkeys. Epidemiology and Infection. 141, 1286-1297. https://doi.org/ 10.1017/ S0950268812001811.

Babaei L, Sharifani MM, Darvishzadeh R, Abbaspour N, Henareh M (2021) Impact of drought stress on photosynthetic response of some pear species. International Journal of Horticultural. Science and Technology. 8, 353-69. https://doi.org// 10.22059/ijhst. 2020.309629.394.

Baltasar Z, Verdonk JC (2022) RNA Extraction from Plant Tissue with Homemade Acid Guanidinium Thiocyanate Phenol

Chloroform (AGPC). Current Protocols. 2, e351. doi: 10.1002/cpz1.351.

Behzadi Rad P, Roozban MR, Karimi S, Ghahremani R, Vahdati K (2021) Osmolyte accumulation and sodium compartmentation has a key role in salinity tolerance of pistachios rootstocks. Agriculture. 11(8), 708.

Chang E, Zhao Y, Wei Q, Shi S, Jiang Z (2016) Isolation of high-quality RNA from Platycladus orientalis and other Cupressaceae plants. Electronic Journal of Biotechnology 23, 21–27. https://doi.org//10.1016/j.ejbt.2016.08.003

F Faostat (2016) FAO web page. http://www.fao.org/faostat.

Gangwar M, Alka Sh, Virendra K, Pradyot P (2021) Assessment of Successful qRT-PCR of SARS-CoV-2 Assay in Pool Screening Using Isopropyl Alcohol Purification Step in RNA Extraction. BioMed Research International. https://doi.org//10.1155/2021/6653950.

Hosseini N, Rezanejad F, Zamani Bahramabadi E (2022) Effects of Soil Texture, Irrigation Intervals, and Cultivar on some Nut Qualities and Different Types of Fruit Blankness in Pistachio (*Pistacia vera* L.). International Journal of Horticultural Science and Technology. 9, 41-53. https://doi.org//10.22059/ijhst.2020.303725.374.

Honaas L, Kahn E (2017) A practical examination of RNA isolation methods for European pear (Pyrus communis). BMC Research Notes 10:237. https://doi.org/10.1186/s13104-017-2564-2.

Honeywooda MJ, Milesb SJ, Wonga K, Harringtonb ChB, Cara C, Obersteb MS, Bowenb Michael D, Vegab Everardo (2022) Use of guanidine thiocyanate-based nucleic acid extraction buffers to inactivate poliovirus in potentially infectious materials. Journal of Virological Methods. 297, 114262. https://doi.org/10.1016/j.jviromet.

Liao X, Zhao Y, Li H, Hou W, Tang X, Zhou R (2023) A simple and rapid method for isolating high-quality RNA from kenaf with high polysaccharide and polyphenol contents. BioTechniques 75, 218–226. https://doi.org/10.2144/btn-2023-0077.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25 p. 402-8. ,https://doi.org/10.1006/meth.2001.1262.

Moazzzam Jazi M, Seyedi SM, Ebrahimie E, Ebrahimi M, De Moro G, Botanga Ch (2017) A genome-wide transcriptome map of pistachio (*Pistacia vera* L.) provides novel insights into salinity-related genes and marker discovery. BMC Genomics 18:627. https://doi.org/10.1186/s12864-017-3989-7.

Moazzam Jazi M, Rajaei S, Seyedi SM (2015) Isolation of high-quality RNA from pistachio (*Pistacia vera* L.) and other woody plants high in secondary metabolites. Physiology and Molecular Biology of Plants. 21, 597–603. https://doi. org/ 10.1007/s 12298-015-0319.

Majidi M, Bahmani Y (2018) Isolation of high-quality RNA from a wide range of woody plants. Journal of Plant Molecular Breeding. 5, 50-59. https://doi.org/10.22058/JPMB.2018.82001.1157.

Mohit Rabari K, Roozban MR, Souri MK, Sadeghi-Majd R, Hamedpour-Darabi M, Vahdati K (2023) Exogenous calcium improves growth and physiological responses of pistachio rootstocks against excess boron under salinity. Journal of Plant Nutrition, 46(17), 4252-4266.

Morantel CJ, Marchart SS, Márquez MA, Martínez EM, Luque I (2014) RNA isolation from

loquat and other recalcitrant woody plants with high quality and yield. Analytical Biochemistry. 452, 46–53. https://doi.org /10.1016/j.ab.2014.02.010.

Nadiya F, Anjali N, Gangaprasad A, Sabu KK (2015) High-quality RNA extraction from small cardamom tissues rich in polysaccharides and polyphenols. Analytical Biochemistry 15:25–7. https://doi.org/10.1016/j.ab.2015.05.017.

Nazoori F, ZamaniBahramabadi E, Mirdehghan SH, Yousefi M (2022) Preharvest application of sulfur as Pesticide on Fresh Hull and Kernel of Pistachio (*Pistacia vera* L.). International Journal of Horticultural Science and Technology 9, 117-29. doi. org/10. 22059 / ijhst. 2021.313206.408.

Nazoori F, ZamaniBahramabadi E, Rezaei H (2024) Effect of sun-drying and roasting on pistachio quality and health benefits. International Journal of Horticultural Science and Technology. 11, 299-308. https://doi.org/10.22059/ijhst.2023.358161.635

Norozi M, ValizadehKaji B, Karimi R, Nikoogoftar Sedghi M (2019) Effects of foliar application of potassium and zinc on pistachio (*Pistacia vera* L.) fruit yield. International Journal of Horticultural Science and Technology 6, 113-23. https://doi.org/10.22059/ijhst.2021.313206.408.

Seçgin Z, Gökhan G, Elif SA, Kızıldoğan KA, Kavas M (2020) Development of new total RNA isolation method for tissues with rich phenolic compounds. Turkish Journal of Biochemistry 45, 343–350. https://doi.org/ 10.1515/tjb-2019-0375.

Roozban MR, Mohamadi N and Vahdati K (2006) Fat content and fatty acid composition of four Iranian pistachio varieties grown in Iran. Acta Horticulturae. 726, 573-577.

Sharifkhah M, Bakhshi D, Pourghayoumi M, Abdi S, Hokmabadi H (2020) Effect of pollination time on yield and antioxidant properties of some pistachio cultivars. International Journal of Horticultural Science and Technology. 7, 51-58. https://doi. org/10.22059/ijhst.2020.285527.306

Tang D, Wei F, Kashif MH, Munsif F, Zhou R (2019) Identification and analysis of RNA editing sites in chloroplast transcripts of kenaf (*Hibiscus cannabinus* L.). 3 Biotech. 9(10), 361. https://doi.org/10.1007/s13205-019-1893-3.

Toni LS, Garcia AM, Jefrey DA, Jiang X, Stauffer B (2018) Optimization of phenol-chloroform RNA extraction, MethodsX 5, pp. 599–608. https://doi.org/10.1016/j.mex.2018.05.011.

Xiao Y, Yang Y, Cao H, Fan H, Ma Z, Lei x, Mason A.S, Xia Z, Huang X (2012) Efficient isolation of high quality RNA from tropical palms for RNA-seq analysis. Plant Omics. 5, 584–558.

Yee W, Kumar JN, Muthusamy PD (2018) Inclusion of 2-Mercaptoethanol in Lysis Buffer Could Interfere with Isolation of High Molecular Weight DNA from Freshwater Microalgae. Indian Journal Microbiology. 58,109–113. https://doi.org/ 10.1007/s12088-017-0698-5.