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A Simple and Efficient Protocol of RNA Extraction from Apple Leaves (*Malus x domestica*); A Silica Column-Based Method

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ABSTRACT

Extracting total RNA from plant tissue using the conventional isolation method is time-consuming, requires expensive reagents and produces low-quality RNA. Over time, researchers have used many commercial RNA extraction kits on silica column-based to obtain high-quality RNA so that the isolated RNA can further use for sequencing and quantitative PCR analysis. This study aimed to identify the efficient apple leaf extraction method with high concentration and good quality RNA extraction. In this study, we applied four isolation kits to extract RNA from apple leaves, such as NucleoSpin® RNA Plant Kit (Macherey-Nagel), Quick-RNA™ Miniprep Kit (Zymo Research), PureLink® RNA Mini Kit (Ambion), and RibospinTM Plant Kit (GeneAll). The results showed that apple leaves RNA isolation successfully using the Ribospin™ Plant Kit (Geneall) reagent with a concentration of 59.9±12.3 ng/µl in good RNA integrity. So, these isolated RNA can be used for further analysis.

Keywords:
Apple Leaves; RNA Extraction; RNA Quality; Silica-column

1. Introduction

Apple (*Malus x domestica*) is a primary cultivated temperate fruit. Regarding genomic evidence, apples were transported from Central Asia into West Europe along the Silk Road and then created cultivated apples because of hybridization and introgression (Cornille et al., 2014). In order to be planted in tropical regions like Indonesia, it would be more suitable to be planted in mountainous areas. Apple plants can optimally grow at an altitude of 1000 - 1500 m above sea level. Malang Raya is Indonesia's apple production center (Sutopo, 2015). Increasing temperature due to global warming causes the productive land for apple cultivation is decreasing. Screening candidates in apple breeding is not only a costly but also long terms process (Jung et al., 2020). An alternative strategy is to plant new clones tolerant to high temperatures, low soil

fertility, and pest and disease attacks. Mutation breeding through tissue culture and DNA markers are technologies that can be done to get the expected results.

An appropriate RNA isolation method is required to obtain high-quality and quantity RNA. Many researchers have recently used the commercial kit for nucleic acid (DNA, RNA) extraction methods. Hu et al. (2020) stated that the extraction methods are divided into liquid-phase extraction and solid-phase extraction. First is liquid phase extraction, sometimes called the conventional method. The acid guanidinium thiocyanate-phenol-chloroform (AGPC) methods use organic solvents and salts for precipitating and separating RNA from the liquid phase. Second, solid-phase extraction methods are performed mainly by using a spin column. The interactions in solid phase extraction are based on liquid and stationary phases, functional groups of nucleic acids and solid sorbents, which selectively separate the target from the solution (Ali et al., 2017). In addition, Das et al. (2020) mentioned three categories of RNA extraction methods: organic extraction, followed by RNA precipitation, purification using a silica-membrane column, and silica-coated magnetic beads/particles. Among several types of RNA extraction kits available in the market, silica column-based is one of the most preferable, because it's simple, rapid and less contamination. Sabat et al. (2021) stated that all kits have the same rules and protocols: inhibiting ribonuclease (RNase P) activity after cell lysis, minimizing genomic DNA existences, and recovering the pure RNA.

Extracting the total RNA from plant tissues with high secondary metabolites is challenging. The biggest challenge in RNA extraction is to eliminate the phenolic compounds. Phenolics bind to nucleic acids by forming high-molecular-weight complexes that can decrease their yield (Fort et al., 2008). The extraction kits are time-efficient; they are only sometimes made for polyphenol- and polysaccharide-rich plant tissues (De Wever et al., 2020). Moreover, a unique handling technique is required during RNA extraction due to the presence of RNases, which make RNA unstable and susceptible to degradation.

Up to now, several researchers have carried out the extraction of RNA from apple plant tissue. RNA bud, internodal shoot, flower, and fruit tissues of apple have been isolated by Gasic et al. (2004) using cetyltrimethylammonium bromide (CTAB)-based methods for making cDNA library construction. El-Sharkawy et al. (2015) isolated RNA from apple fruit skin for transcriptomic analysis to identify anthocyanin-related genes and epigenetic regulation. In addition, Zhu et al. (2017) and Tian et al. (2019) have succeeded in isolating RNA from apple leaf, which is the first step in the transcriptomic analysis research used for research in apple defense against the fungus and resistance to powdery mildew (PM) respectively. Zhu et al. (2017) used CTAB-based methods like Gasic et al. (2004). El-Sharkawy et al. (2015) and Tian et al. (2019) used column-based RNA isolation kits, namely the RNeasy Mini Kit (Qiagen) and EZNA Plant RNA Kit (Omega Bio-Tek). Recently, Nabi et al. (2022) reported that they isolated RNA from apple leaves to show mosaic/necrotic mosaic using the Qiagen RNeasy® Plant Minikit.

Recently, a large number of nucleic acid isolation methods have been modified, such as CTAB, SDS, commercial kits methods and so on. Due to the different composition of samples, especially in plants which are rich in polyphenols, polysaccharides, and secondary metabolites, make the extraction methods vary. Many publications reported successful RNA isolation methods for different species with certain conditions. Azizi et al. (2017) evaluated five different methods which used to isolate total RNA for Malaysian rice varieties leaves, methods 1 (CTAB), method 2 (SDS), method 3 (TRIzol),

method 4 (Invitrogen), method 5 (RNeasy plant mini kit). The TRIzol showed the highest concentration and A260/280 ratios 1781 µg/µL and 2.20 respectively. Carpinetti et al., (2021) studied six extraction protocols, two protocols are CTAB-based methods, one which applies guanidine thiocyanate followed by organic phase extraction, TRIzol reagent, PureLink RNA Mini Kit and RNeasy Plant Mini Kit, for extracting RNA from *Psidium guajava* L. leaves. CTAB-based method provided the highest RNA yields and quality. The RNA samples from CTAB2 were 676.87 µg/µL, and the A260/A280 and A260/A230 ratios were 2.1 and 2.2, respectively. The CTAB-based method has also been used successfully for isolating RNA by Guzman et al. (2014) in *Eugenia uniflora*, and Vining et al. (2015) and Favreau et al. (2019) in *Eucalyptus grandis*, to perform transcriptome and gene expression analyses.

Even though many commercial kits are available in the market, optimization of RNA extraction using silica column-based methods for apple (*Malus x domestica*) leaves has yet to be studied thoroughly. This study aimed to identify the most efficient method of four silica column-based kits commercially available for extraction from apple tissue with high concentration and good quality RNA extracted.

2. Materials and Methods

2.1 Plant materials

Apple leaves were collected from apple plantations in Dusun Kunci Desa Wringinanom, Poncokusumo, Malang Regency, East Java. The young leaves of apple were cut from the plant, and immediately washed with water then cut into pieces and stored in RNA*later* Stabilization (Sigma Aldrich) solution before being used. Incubation with RNA later Stabilization Solution according to the manufacturer's protocol. When the sample is ready to isolate the RNA, remove the RNA*later* and let the leaf dry. Mortars, pestles, scissors, and all ware were autoclaved before used and then treated with RNaseZAP™ (Sigma Aldrich). Filter tips and tubes were RNase and DNase-free.

2.2 RNA Isolation Methods

Four commercial kits for RNA extraction, such as NucleoSpin® RNA Plant (Macherey-Nagel, 2019), Quick-RNA™ Miniprep Kit (Zymo Research, 2003), PureLink® RNA Mini Kit (Ambion, 2010), and Ribospin™ Plant (GeneAll) were tried for RNA isolation. The all methods were performed according to the manufacturer's guide protocol without modification.

2.2.1 NucleoSpin® RNA Plant (Macherey-Nagel, 2019)

Samples (up to 100 mg) were ground under liquid nitrogen. Subsequently, 350 µL of Buffer RA1 and 3.5 µL of ß-mercaptoethanol (ß-ME) were added to 100 mg tissue and vortex vigorously. The lysate was filtrated through the NucleoSpin® Filter (violet ring). NucleoSpin® Filter was placed in a collection tube (2 mL), and then a mixture was applied and centrifuged at 11,000 xg for 1 minute. The filtrate was transferred to a new 1.5 mL microcentrifuge tube. NucleoSpin® Filter was discarded, and 350 µL of ethanol (70%) was added to the homogenized lysate and then mixed by pipetting up and down. NucleoSpin® RNA Plant Column (light blue ring) was placed in a collection tube, and the lysate was loaded. The lysate was then centrifuged for 30 seconds at 11,000 xg. The column was placed in a new collection tube (2 mL). Into the tube, three hundred and fifty µL MDB (Membrane Desalting Buffer) was added and centrifuged at 11,000 xg for 1 minute to dry the membrane. Next, the DNase reaction mixture was prepared in a sterile 1.5 mL microcentrifuge tube with the addition of 10 µL of

reconstituted rDNase and 90 µL of Reaction Buffer for rDNase, then 95 µL of the DNase reaction mixture was applied directly onto the center of the silica membrane of the column. After that, the mixture was incubated at room temperature for 15 minutes. Buffer RAW2 (1st wash), 200 µL, was added to the NucleoSpin® RNA Plant Column. And then, centrifuged for 30 seconds at 11,000 xg. The column was placed into a new collection tube of 2 mL. Six hundred µL of Buffer RA3 (2nd wash) was added to the NucleoSpin® RNA Plant Column. And then, centrifuged for 30 seconds at 11,000 xg. Flow through was discarded, and the column was placed back into the collection tube. Around 250 µL of Buffer RA3 (3rd wash) was added to the NucleoSpin® RNA Plant Column and centrifuged for 2 minutes at 11,000 xg to dry the membrane completely. The column was placed into a nuclease-free collection tube. The RNA was eluted in 60 µL of RNase-free H_2O and centrifuged at 11,000 xg for 1 minute. The eluted RNA was stored at -70°C until further use.

2.2.2 Quick-RNA™ Miniprep Kit (Zymo Research, 2003)

Plant tissue (up to 100 mg) was homogenized, and then 600 µL of RNA Lysis Buffer was added and mixed by vortexing. The lysate was centrifugated at 11,000 xg for 1 minute. The supernatant was transferred into a Spin-Away™ Filter (yellow) in a collection tube and then centrifuged at ≥11,000 xg for 1 minute to remove the majority of gDNA. The flow-through was collected. One volume of ethanol absolute was added and then mixed well. The mixture was transferred to a Zymo-Spin™ IIICG. Column (green) in a collection tube and centrifuged at 11,000 xg for 1 minute. The flow-through was discarded. DNA. Digestion Buffer and DNase I was mixed, 5 µL and 75 µL respectively, then added to the matrix column and incubated in T room (20-30°C) for 15 minutes. Four hundred µl of RNA Prep Buffer was added to the column and centrifuged at 11,000 xg for 1 minute. The flow-through was discarded, and then 700 µl RNA Wash Buffer was added to the column and centrifuged at 11,000 xg for 1 minute. The flow-through was discarded. Four hundred µl of RNA Wash Buffer was added and centrifuged in the column at 11,000 xg for 2 minutes to ensure complete removal of the wash buffer. The column was transferred carefully into an RNase-free tube. Fifty µl of DNase/RNase-Free Water was added directly to the column matrix and centrifuged at 11,000 xg for 1 minute. The eluted RNA was stored at -70°C until further use.

2.2.3 PureLink® RNA Mini Kit (Ambion, 2010)

Samples were ground into a fine powder using a mortar and pestle with liquid Nitrogen, and the ground sample was transferred into a 1.5 ml microcentrifuge tube. Immediately add the appropriate volume of Lysis Buffer prepared with 2–mercaptoethanol to the sample. The sample was homogenized using a rotor-stator at maximum speed for at least 45 seconds, then centrifuged at ~2,600 × g for 5 minutes at room temperature. The supernatant was transferred to a new RNase-free tube. Half of the 96–100% ethanol was added to each volume of tissue homogenate and mixed by vortexing thoroughly. Around 700 µL of the sample was transferred to the spin cartridge (with a collection tube) and then centrifuged at 12,000 ×g for 15 seconds at room temperature. The flow-through was discarded, and reinsert the spin cartridge in the same collection tube, then repeated steps until the entire sample was processed. Then 700 µL of Wash Buffer I was added to the spin cartridge and centrifuged at 12,000 ×g for 15 seconds at room temperature. The flow-through was discarded, and the spin cartridge was placed into a new collection tube. Five hundred µL of Wash Buffer II was applied to the spin cartridge and followed by ethanol. The mixture was centrifuged at 12,000 ×g for 15 seconds at room temperature. The flow-through was discarded, and

the spin cartridge was reinserted in the same collection tube. Steps were repeated (500 µL Wash Buffer II was added with ethanol to the spin cartridge, centrifuged at 12,000 ×g for 15 seconds at room temperature and the flow-through was discarded), and the spin cartridge was reinserted in the same collection tube. The spin cartridge with a collection tube was centrifuged at 12,000 × g for 1 minute at room temperature to dry the membrane with an attached RNA The collection tube was discarded, and the spin cartridge was inserted into a recovery tube. Approximately 30 µL –100 µL of RNase-Free Water was added to the center of the spin cartridge and then incubated at room temperature for 1 minute. The spin cartridge was centrifuged for 2 minutes at ≥12,000 ×g at room temperature, and the purified RNA was stored at -70^{0}C until further use.

2.2.4 Ribospin™ Plant (Geneall, 2012)

Sample (up to 100 mg) were ground into a fine powder using a mortar and pestle with liquid Nitrogen, and the ground sample was transferred into a 1.5 ml microcentrifuge tube. Three hundred and fifty µL of Buffer R.P.L. was added to the 1.5 ml microcentrifuge tube and vortex vigorously, then incubated for 3 minutes at room temperature. The lysate was transferred to an EzPureTM filter and centrifuged at ≥ 10,000 xg for 30 seconds at room temperature. The supernatant was transferred to a new 1.5 ml microcentrifuge tube. A volume (usually 350 µL) of 70% ethanol was added to the supernatant tube and mixed well by pipetting. The mixture was applied to a mini spin column (type W, blue ring) and centrifuged at ≥ 10,000 xg for 30 seconds at room temperature. The pass-through was discarded, and the mini-spin column was put back into the same tube. Five hundred µL of buffer RBW was added to the mini spin column and centrifuged at ≥ 10,000 xg for 30 seconds at room temperature. The pass-through was discarded, and the mini-spin column was reinserted into the same tube. Seventy µL of DNase I (mix two µl DNase I with 70 µl Buffer D.R.B.) reaction mixture was applied to the center of the mini spin column and incubated at room temperature for 10 minutes. Five hundred µL of buffer RBW was added to the mini spin column and stood for 2 minutes. Centrifuge at ≥ 10,000 xg for 30 seconds at room temperature. The pass-through was discarded, and the mini-spin column was reinserted into the same tube. Five hundred µL of Buffer R.N.W. was added to the mini spin column and centrifuged at ≥ 10,000 xg for 30 seconds at room temperature. (The pass-through was discarded, and the mini spin column was reinserted into the same tube). Then 500 µL of Buffer R.N.W. was added to the mini spin column and centrifuged at ≥ 10,000 xg for 30 seconds at room temperature. The pass-through was discarded, and the mini-spin column was reinserted into the same tube and centrifuged at ≥ 10,000 xg for an additional 1 minute at room temperature to remove residual wash buffer. The mini spin column was transferred to a new 1.5 ml microcentrifuge tube. Thirty µL of RNase-free water was added to the center of the membrane in the mini spin column and then centrifuged at ≥ 10,000 xg for 1 minute at room temperature—purified RNA stored at -70°C.

2.3 Total RNA Quantity and Quality Check

The quality and integrity of extracted total RNA from four different methods were analyzed by running in 1% agarose gel electrophoresis, volumes of 5 µL RNA were loaded in each lane for gel electrophoresis, and visualizing under UV transilluminator. The concentration and purity of extracted RNA were determined by monitoring the A260/280 and A260/230 ratio using Nanodrop ND 1000 Spectrophotometer (NanoDrop Technologies, USA).

3. Results and Discussion

This study observed four silica column-based kits for RNA extraction, namely NucleoSpin® RNA Plant Kit (Macherey-Nagel, Germany), Quick-RNA™ Miniprep Kit (Zymo Research), PureLink® RNA Mini Kit (Ambion), and Ribospin™ Plant Kit (GeneAll). Many publications reported successful RNA isolation methods for different species with certain conditions. A guanidine thiocyanate (GuCN)-phenol-chloroform extraction is the most classical method of RNA isolation (Chomzynski, 1987); this method uses hazardous chemicals even though it gives a high yield of RNA. Moreover, using this method is laborious and time-consuming.

There is an isolation kit for plant RNA available commercially. Still, they can only sometimes be effective for all plant tissues because each kit is designed specifically for the target tissue of the plants. The latest nucleic acid extraction technology (NAE) is based on silica membranes and combined with spin column technology. The advantages of the silica column-based method are simple to set up, rapid (short preparation time, see Table 1), non-toxic, high-purity RNA, and reproducible (Escobar & Hunt (2017); Das et al. (2020)). Yang et al. (2017) also reported that the RNA extracted using silica column-based was less contaminated by polysaccharides, proteins, phenolic compounds, or other reagents.

Table 1. Comparison of RNA extraction method

Kit Name	Manufacturer	Processing time	Spin speed required	Sample material	Maximum binding capacity
NucleoSpin® RNA Plant	Macherey-Nagel	30 min/6 preps	11,000 xg	< 100 mg tissue	200 µg
Quick-RNA™ Miniprep Kit	Zymo Research	Less than 30 minutes	≥10,000 xg	tissue samples (up to 50 mg)	n.a
PureLink® RNA Mini Kit	Ambion	Less than 1 hour	12,000 × g	≤ 250 mg plant tissue	~1 mg nucleic acid
Ribospin™ Plant	GeneAll	25 minutes	≥10,000 xg	100 mg plant tissue	~ 100 µg

The four kits were a spin-column-based method. Like the other DNA/RNA isolation methods, four key steps involve in DNA/RNA extraction cell lysis, nucleic acids adsorption/binding, washing, and elution (Lem, 2020) (Figure 1). During lysis, the cells are broken to allow the nucleic acid to be released into the solution. The spin column is used in the binding step, with the binding solution applied to the column. The spin columns contain a silica resin that selectively binds nucleic acids. Those purification methods use chaotropic salts to denature proteins (including DNases and RNases) and denature nucleic acids by disrupting their hydrogen bonding. This protocol provides the selective binding of nucleic acids to the silica resin in the column. The nucleic acids are washed with chaotropic salts to remove protein and pigment contaminants and ethanol to remove salts. After washing, nucleic acids are eluted from the column with water or low-salt solution, which induces its renaturation and thus eliminates their affinity for the silica resin (Yaffe et al., 2012). Eluted nucleic acid was then collected at the bottom of the tube. Moreover, in general, the process of RNA extraction can be completed less than an hour (Table 1), which is time-saving.

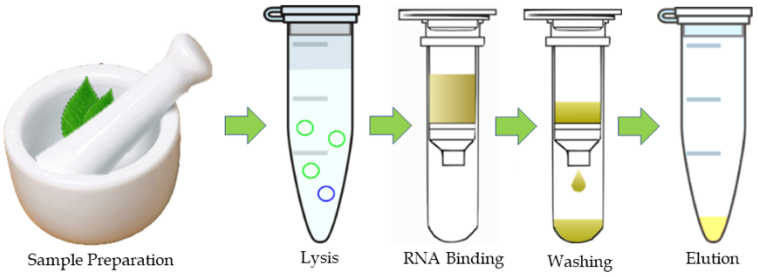

Figure 1. Overview of steps in RNA extraction protocol

The method for assessing RNA concentration and purity is determined by measuring the absorbance of ultraviolet light (Koetsier & Cantor, 2019). The ratio of absorbance at 260 nm and 280 nm is used to assess RNA purity. The A260/A280 ratio ~2.0 is generally accepted as pure for RNA (ThermoScientific, 2011). In addition, Koetsier & Cantor., (2019) mentioned that RNA samples with A260/A280 and A260/A230 >1.8 are considered to be clean, and suitable for use in most downstream applications. The 260/230 values are often higher than the 260/280 values. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm. The 260/230 ratio is used to indicate the presence of unwanted organic compounds such as Trizol, phenol, Guanidine HCL and guanidine (Denovix, 2022).

The concentrations of total extracted RNA are presented in Table 2, the Quick-RNA™ Miniprep Kit (Zymo Research) gave the highest concentration of 141.5 ng/µl compared to the other three kits, and RNA extracted by using NucleoSpin® RNA Plant kit obtained low RNA concentration of 6.54 ng/µl. It was followed by PureLink® RNA Mini Kit (Ambion, 2010), which produce an RNA concentration of around 7.62 ng/µl. Meanwhile, isolation using Ribospin™ Plant (Geneall) produced RNA of about 59.9 ng/µl. This concentration is sufficient as a DNA template for PCR amplification, because concentration requirement of template DNA is between 1 ng to 1 µg (Biolabs, 2023).

Besides the high concentration of RNA extracted that we target, there are two other important parameters for evaluating that RNA extracted can be used for further application; purity and integrity. The pure RNA is indicated by the ratio of absorbance (A) ratio at 260 nm and 280 nm with the value ~2.0. If the ratio is lower than 2.0, this may exhibit the presence of contaminants that absorb at 280 nm (ThermoScientific, 2011). The good RNA integrity can be seen from the distinct 28S and 18S ribosomal RNA (rRNA) bands in RNA extracted.

Based on Table 2, the comparison of A260-280 in all samples is in the range of 1.86 to 1.98 except for PureLink® RNA Mini Kit (Ambion, 2010), which is only 1.56. This A260-280 ratio of 1.8-2.0 indicates that the sample has no protein contamination. Meanwhile, the ratio of A260/230 was 1.53 for Ribospin™ Plant (GeneAll), while for the three other RNA extraction kits, the comparison of A260/230 showed a value of < 1. The low ratio of A260/230 indicates the presence of contamination that absorbs at a wavelength of 230 nm. These contaminants are significantly more numerous than those absorbing at 280 nm. Koetsier & Cantor., (2019) mentioned that it could be interference by chaotropic salts such as guanidine thiocyanate (G.T.C.) and guanidine hydrochloride (GuHCl), EDTA, non-ionic detergents like Triton™ X-100 and Tween® 20, proteins, and phenol. Chaotropic salts, like G.T.C. and GuHCl, are frequently used

in binding buffers. Non-ionic detergents, such as Triton X-100 and Tween 20, are often found in lysis buffers.

Table 2. Concentration and purity of RNA isolated from apple leaf using different methods

Extraction method	Concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
NucleoSpin® RNA Plant	6.54±1.80	1.86±0.26	0.10±0.04
Quick-RNA™ Miniprep Kit (Zymo Research)	141.4±79.7	1.90±0.06	0.98±0.26
PureLink® RNA Mini Kit (Ambion)	7.62±2.36	1.56±0.13	0.29±0.09
Ribospin™ Plant (Geneall)	59.9±12.3	1.98±0.06	1.53±0.19

Note: N = 3 sample replicates per extraction kit.

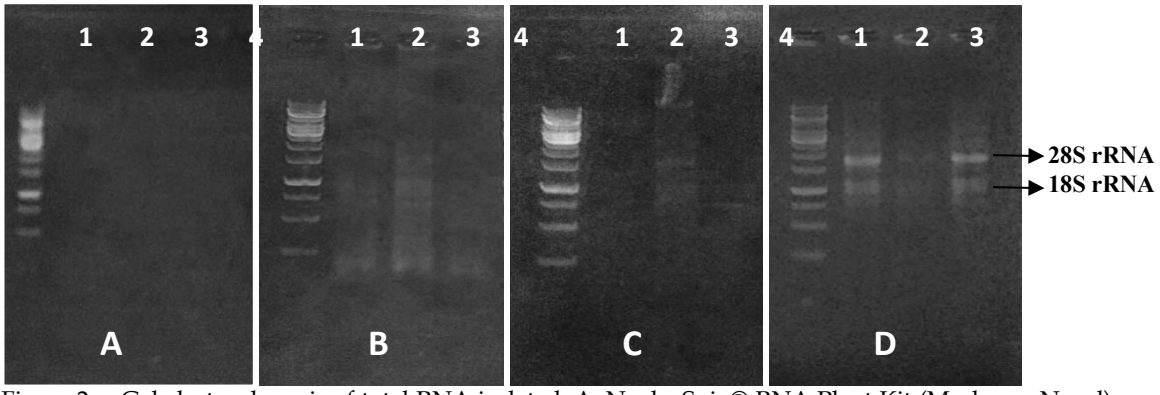

Figure 2. Gel electrophoresis of total RNA isolated. A. NucleoSpin® RNA Plant Kit (Macherey-Nagel); B. Quick-RNA™ Miniprep Kit (Zymo Research); C. PureLink® RNA Mini Kit (Ambion); D. Ribospin™ Plant (Geneall) kit. 1: DNA. ladder 1 kb; 2: first RNA sample; 3: second RNA sample; 3: third RNA sample.

The second parameter is the integrity of the RNA extracted. It was evaluated by 1% of agarose gel electrophoresis. Figure 2 shows that extraction using GeneAll RNA, the good intact RNA was observed with two bright bands (28S rRNA and 18S rRNA) (Figure 2D). The results of this band show the brightest bands than the other three kits. However, Quick-RNA™ Miniprep Kit (Zymo Research, 2003) presented the gel electrophoresis, which visualizes the blurred band of RNA (Figure 2B). The blurred band showed the RNA was degraded. Moreover, the PureLink® RNA Mini Kit (Ambion, 2010) shows a similar case to Quick-RNA™ Miniprep Kit (Figure 2C). The bands are not as sharp as the bands in Figure 2D. On the contrary, in Figure 1A, no bands were visible in RNA extracted using NucleoSpin® RNA Plant; it is suspected that the RNA cannot be extracted properly using this kit, so that it shows the lowest concentration.

With these results, using Ribospin produces good results based on the concentration, purity and integrity of RNA So, RNA can be used for the next step of sequencing using Illumina Stranded mRNA Prep because it has a sufficient amount to meet the requirement of input quantity of about 25-1000 ng total RNA (Illumina, 2021). The consistent results were also shown by Zulaeha et al., (2019), where isolation of RNA

from leaves, callus and somatic embryos of oil palm using Ribospin™ was better than using Plant RNA PureLink (Ambion) and Genezol RNA Extraction (Geneaid). The total RNA concentration obtained from the previous study was higher than 4 µg, and the RNA Integrity Number (RIN) value was more than 7.

4. Conclusion

In this study, Ribospin™ Plant Kit (Geneall, 2012) method produces a high concentration, and high-quality total RNA of apple leaves around the concentration of 59.9 ng/µl with good integrity of RNA. This method is suitable for further molecular applications.

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