ISOLATION OF TOTAL RNA AT EARLY STAGE OF JATROPHA SEEDS DEVELOPMENT

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DECLARATION

I hereby solemnly and sincerely declare that the project work entitled "Isolation of Total RNA at Early Stage of Jatropha Seeds Development" submitted to the Faculty of Resource Science and Technology, University Malaysia Sarawak is a presentation of my original research work and that it has not been submitted anywhere for any award. The work was done under the guidance of Madame Safarina Ahmad and this report work is submitted in the partial fulfillment of the requirement for the award of the Bachelor of Science with Honours in Resource Biotechnology.

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LIST OF ABBREVIATIONS

cDNA	Complementary deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamineetetraacetic
EtBr	Ethidium bromide
FAME	Fatty acid methyl esters
FFA	Free acid methyl esters
NaOH	Sodium hydroxide
PVP	Polyvinypyrrolidone
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolutions per minute
RT-PCR	Reverse transcription- Polymerase Chain Reaction
tRNA	transfer RNA
TAG	Triacylglycerol

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Isolation of Total RNA at Early Stage of Jatropha Seeds Development

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ABSTRACT

Jatropha curcas is an oil-bearing shrub and has great potential for development as a biofuel crop. Genetic engineering technology is being adopted to improve the production and quality of the biofuel. *J. curcas* seed is rich in fatty acid, storage protein, polysaccharides and other secondary metabolites which make isolation of total RNA to be difficult. The aim for this study is to generate an effective protocol which able to isolate good quality of total RNA from early stage of Jatropha seeds that is very important for many gene expression studies. The described methods were CTAB-LiCl method I, CTAB-LiCl II and CTAB-isopropanol method. Compare to these three methods, CTAB-LiCl method II was the preferred method. This method was able to yield up to 0.239 µg/µl of RNA with the A_{260}/A_{280} and A_{260}/A_{230} ratio ranged from 1.8 - 2.0 and 0.619 - 1.248, respectively. This indicated total RNA free of protein contamination while slight contaminated with organic compound. Using the CTAB-LiCl method II, RNA was successfully obtained from Jatropha seeds.

Keywords: *Jatropha curcas*, CTAB- LiCl method, CTAB- isopropanol method, *total* RNA isolation

ABSTRAK

Jatropha curcas merupakan tumbuhan semak berkayu yang mempunyai kandungan minyak yang tinggi. J. curcas mempunyai potensi besar untuk dikembangkan sebagai sumber biofuel. Teknologi kejuruteraan genetik digunakan untuk meningkatkan pengeluaran dan kualiti biofuel. Pemencilan RNA yang berkualiti tinggi daripada biji J. curcas adalah sukar disebabkan kehadiran asid lemak, protein simpanan, polisakarida dan metabolit sekunder yang lain yang tinggi. Kajian ini dilakukan untuk menghasilkan protokol yang berkesan untuk memencilkan jumlah RNA yang berkualiti tinggi dari biji Jatropha. Kaedah yang berkesan adalah penting dalam kebanyakkan kajian ekspresi gen. Kaedah yang digunakan ialah kaedah pemencilan CTAB- LiCl I, CTAB-LiCl II dan CTAB-isopropanol. Antara ketiga-tiga kaedah ini, kaedah pemencilan CTAB-LiCl II merupakan kaedah yang paling sesuai untuk pemencilan RNA. Kaedah ini dapat menghasilkan sebanyak 0.239 µg/µl RNA dengan nisbah A_{260}/A_{280} dan A_{260}/A_{230} berkisar dari 1.8 - 2.0 dan 0.619 - 1.248 masing-masing. *Ini menunjukkan bahawa RNA bebas dari pencemaran protein tetapi tercemar dengan sebatian organik. Dengan menggunakan kaedah CTAB-LiCl II, RNA telah berjaya dipencilkan dari biji Jatropha.*

Kata kunci: Jatropha curcas, kaedah CTAB-LiCl, kaedah CTAB-isopropanol, pemencilan RNA

1.0 INTRODUCTION

Jatropha curcas is locally known as "jarak pagar" in Malaysia, especially in Peninsular Malaysia (Salimon & Abdullah, 2008). It is belonging to tribe Joannesieae Crotonoideae in the Euphorbiaceae family (Divakara *et al.*, 2010). *J. curcas* was first named by Linnaeus (1753) according to the binomial nomenclature of "Species Plantarum" (Heller, 1996). The term *Jatropha* is derived from Greek word "jatro" (doctor) and "trophe" (food/ nutrition) which implies its medical use (Divakara *et al.*, 2010). It is a native to tropical American but now thrives in many Latin American, Asian and African countries (Openshaw, 2000). Although Jatropha seed has high nutritional value since its kernel contains 22-27 % protein and 57-63 % lipid (Makkar & Becker, 1997), its toxic content especially "phorbol ester" cause it not edible and limited as food or feed source.

In some developing country, shortage of edible oil for human consumption will lead it not suitable for bio-diesel production. The non- edible oil from *J. curcas* seeds can be considered as a suitable source for the production of high quality biodiesel (Ye, *et al.*, 2009). Nowadays, biofuel is increasingly developing in Asia. Thailand and Indonesia also focus on the use of Jatropha oil as biofuel besides using the palm oil. Although government and private sectors in Malaysia have shown their interest in the developing of Jatropha oil, it is still in the nascent state (Lim & Teong, 2010). To date, the demand for biofuel production is keep on increasing. This urged the researchers to explore the oil rich Jatropha through biotechnology in order to understand the molecular basis in the seed oil improvement.

In this study, total RNA is isolated from early stage of Jatropha seeds because it is the important initial step for many molecular studies in plant biology, such as cDNA library construction. However, Jatropha seeds contain high quantity of polysaccharides, carbohydrates, lipids, polyphenols and proteins in its tissues which can interfere the

successful extraction of total RNA. This may due to the co-purification of these secondary metabolites with isolated RNA. Polysaccharides and polyphenolics will bind to the RNAs during extraction to form the viscous, insoluble complexes that will eventually interfere the isolation steps (Pawlowski *et al.*, 1994, cited in Liu *et al.*, 1998). In addition, the instability and susceptibility of RNA to degradation by RNase which can be found anywhere such as skin and glassware is another obstacle for total RNA isolation (Rubio-Pina & Vazquez-Flota, 2008).

In this study, CTAB-LiCl method I, CTAB-LiCl method II and CTAB-isopropanol method were performed on *J. Curcas*. β-mercaptoethanol which is one of the salt in CTAB extraction buffer able to prevent sample oxidation and inhibit RNase release from tissue prior to chloroform extraction (Fu *et al.*, 2004). Moreover, according to Barlow *et al.* (1963), LiCl is a very effective method for precipitating RNA due to it inefficiently precipitates DNA, protein or carbohydrate. In addition, it removes free nucleotides efficiently to give more accurate value when quantitated by UV spectrophotometry. The success of the total RNA isolation was determined by high quality of total RNA with less contamination of polysaccharides and other secondary metabolites and distinct bands of 28S and 18S rRNA.

The objectives of this study are:

1. To isolate high quality and quantity of total RNA from early stage of Jatropha seeds.
2. To develop an effective protocol for total RNA isolation from early stage of Jatropha seeds

2.0 LITERATURE REVIEW

2.1 *Jatropha curcas*

J. curcas is native at Central and South Amerika but now it is widely grown in many tropical and subtropical countries such as Myanmar, Thailand, Laos, Cambodia, India and Malaysia (Ye *et al.*, 2009). Jatropha plants contain approximately 175 species in the world. It is a multipurpose oil-bearing shrub which has life expectancy up to 50 years. In addition, Divakara *et al.* (2010) pointed out that it can grow to a height of about 5 m but if it grows under the favorable conditions, it can reach a height of 8 to 10 m (Figure 2.1).

J. curcas is succulent and able to adapt itself to dry season by sheds its leaves. Since Jatropha is drought resistant species, it able to grow at the marginal land, arid and semi-arid area. Besides, it can be considered as plant material for eco-restoration in all types of wasteland. Thus, it can use to reclaim land and control erosion. Since, *J. curcas* contains toxic compound, it is highly pest resistant and can be grown as live fence in order to contain or exclude farm animal (Salimon & Abdullah, 2008).

Moreover, *J. curcas* is a cross-pollinated crop. There are two propagation method for *J. curcas*, which are generative propagation (seeds) and vegetative propagation (cuttings). Jatropha seed required 10 days for germination while its fruit required 90 days to develop start from flowering until seed mature (Heller, 1996).

Jatropha is a diploid species with 2n=22 chromosome and base number of x= 11 (Divakara *et al.*, 2010; Heller, 1996). Divakara *et al*. (2010) stated that by using traditional sanger sequencing and next generation sequencing, Synthetic Genomic Inc. (SCI) and Asiatic Centre for Genome Technology (ACGT) has revealed that genome of Jatropha is approximately 400 million base pair in size.

Figure 2.1: **Morphology of *J. curcas* tree.** *J. curcas* can grow to a height of about 5 m. However, it can reach a height of 8-10 m if it grows at the favourable conditions. It has smooth bark, papery leaves and sturdy branches. In addition, it also has life expectancy of up to 50 years.
(Source: http://www.ecofriendlymag.com/sustainable-transporation-and-alternative-fuel/the-potential-of-jatropha/)

2.1.1 Lifecycle of Jatropha Fruit

Silip *et al.* (2010) claimed that first buds were appeared around 85 to 98 days after seeding and these buds will develop into flowers within 7 and 18 days. The duration of development of flower to fruits set was taken around 1 to 8 days. From the fruit sets, fruits were developed into mature green stage within 21 to 35 days and become fully yellow or ripe fruit within 2 to 4 days. Lastly, 3 to 9 days is required to allow it to develop into fully senesced fruits (Table 2.1).

2.1.2 Jatropha seeds

Jatropha seeds are black in color with 1.5 to 2 cm long and 5 cm thick (Figure 2.2). Its seed is weigh from 0.4 to over 1 g. Seed oil extracted from Jatropha seed can be used to

produce insecticide, candle, soap, and have medicinal properties. For instance, it can be used to cure disease such as dysentery, hemorrhoids, coated tongue, gonorrhea, infertility, and skin infection (Ahmed & Salimon, 2009).

Table 2.1: Lifecycle duration of *J. curcas* fruit. There are wide variations in days for maturity of fruit, ripening and senescence.
(Source: Silip *et al.*, 2010)

Reprodutive variables	Days		
	Min.	Max.	Average
Seeding to first appearance of bud	85	98	91.5
Bud development	7	18	12.5
Flowering to fruit set	1	8	4.5
Fruit set to physiological mature fruit or mature green	21	35	28
Mature green to yellow fruit	2	4	3
Mature green to black fruit	3	9	6
Mature green to dry fruit	6	17	11.5
Flower to yellow fruit	24	47	35.5
Flower to black fruit	27	56	41.5
Flower to dry fruit	36	73	54.5

Figure 2.2 : Morphology of *J. curcas* seeds. Jatropha seeds are black colour. It is 1.5 to 2 cm long and 5 cm thick.
(Source:http://commons.wikimedia.org/wiki/File:S eed_jatropha_curcas.jpg)

Jatropha seeds are important for the production of biofuel. *J. curcas* oil is suitable for biodiesel production because it contains 78 to 84 % of unsaturated fatty acid (Salimon & Abdullah, 2008). A study by Salimon and Abdullah (2008) found that Jatropha seeds contains more unsaturated fatty acid (78.94 %) compared to the saturated fatty acid (21.05 %). They also pointed out that Jartropha seeds oil extracted from Malaysia consists of oleic 46.00 %, linoleic 31.96 %, palmitic 13.89 %, while less than 10 % is comprised of stearic and palmitoleic.

Although Jatropha seeds responsible in the production of biofuel, its high fraction of the unsaturated fatty acids may influence the oxidation stability of Jatropha oil (Diwani *et al.*, 2009). Seed cake remaining after oil extraction is rich in nitrogen, phosphorus and potash. It is suitable use as organic manure (Janick & Paull, 2008). Seed cake only can use as fodder after processing and detoxification (Openshaw, 2000).

J. curcas seed is toxigenic due to the presence of protein 'curcin' and phorbol ester. However, phorbol esters have been identified as the major toxic compounds in Jatropha (Ahmed & Salimon, 2009). It is a tumour-promoting compound that can regulates different signal transduction pathways and other cellular metabolic activities. Besides that, it also can cause purgation and skin- irritant effect (Garland & Barr, 1998). A study by Ahmed and Salimon (2009) found that Malaysia *J. curcas* seed oil has low level of phorbol esters (0.23 %), compared to Indonesia (1.58 %) and India (0.58 %). However, plant that contains phorbol ester will show it toxicological manifestations in animals' food sources containing them even at very low concentrations (Goel *et al.*, 2007).

2.1.3 Early Stage of Jatropha Seeds

A study about the lipid profiling of developing *J. curcas L*. seeds has been carried out by Annarao *et al.* (2008). They pointed out that early stage of seed has the highest moisture content compared to late stage. Besides that, the seed area which corresponded to increase in fresh weight of the seeds also increases as seed grow and shrink at the late stage. Jatropha seeds will synthesis lipid around three weeks after fertilization whereas synthesis of triacylglycerol (TAG) at the fourth week. As it grows, there is decrease in sterols and free fatty acids (FFA) concentration, increase of TAG accumulation and little fatty acid methyl esters (FAME) formation (Annarao *et al.*, 2008).

Moreover, higher amount of fatty acid and other secondary metabolites which can interfere the total RNA isolation process can be found in Jatropha seeds during maturity (Sangha *et al.*, 2010). Thus, early stage of Jatropha seed that contain less interfering compounds is considered more suitable for total RNA isolation than the late stage of Jatropha seed.

2.1.4 Uses of Jatropha

Besides producing biofuel, it also plays important roles in industrial and medicinal areas. The leaves of *J. curcas* can be traditionally used as an anti-inflammatory and anticoagulant (Nayak & Patel, 2010). Besides, the decoction is use as traditional medicine against coughs, antiseptics after birth, malaria and treats hypertension. Furthermore, the leaf sap can be used to treat haemorrhoids (Richard & NeBambi, 2010).

The stems are chewing sticks in Nigeria that can treat gum disease by strengthen the gum. Moreover, latex produced from the stem can also be used as a medicine by healing the wounds and skin diseases (Richard & NeBambi, 2010). Latex also have

antimicrobial properties which able to against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Streptococcus pyogenes* and *Candida albicans* (Heller, 1996).

2.2 Total RNA

Total RNA is a single stranded molecule that contains all the RNA of the cells, such as mRNA, tRNA and rRNA. Besides, it is suitable for use in gene expression and functional genomics analyses (Baharina, 2005). DNA sequence of gene which passes through RNA intermediate is used to produce specific protein sequence to express their functional effect. Since RNA contains only the functional coding region, analysis of total RNA will provide information about the gene expression, regulation and function. A high-quality and purified total RNA is considered as the starting material for many applications such as Northern hybridization and RT-PCR (Martin *et al.*, 2001).

Moreover, RNA is labile as it is susceptible to degradation by endogenous and exogenous RNases. Unlike DNases, RNases do not require cofactor. Thus, chelating agents cannot eliminate some of the RNase but able to protect DNA against DNases by binding divalent cations (Mark, 1997). Therefore, quality of isolated RNA can improve by carefully handling and protect the tissue or cells from all sources of RNases when storage the samples, RNA isolation process and storage the isolated RNA.

mRNA consists of less than 5 % of the cellular RNA. Small amount of purified mRNA is difficult to quantitative and particular messenger species has the risk to be loss during mRNA purification (Martin *et al.,* 2001). Thus, in this study, total RNA will be isolated from the *J. curcas* seed instead of mRNA. Moreover, the use of RNA instead of DNA in this study is due to the absence of intron in the RNA and its suitability for genomic expression library construction (Baharina, 2005).

2.3 RNA isolation method

Isolating high-quality RNA from plant tissue is often difficult due to large quantities of lipids, polysaccharides, phenols and other secondary metabolites that accumulate in this tissue and co-purify with the RNA. Plant tissue that rich in lipid and polysaccharides such as *J. curcas* seeds will have the risk in difficulty to separate the RNA and the cellular debris. This can be evidenced by the presence of white flocculent material which unable to accumulate as interface in the aqueous phase after centrifugation (Martin *et al.*, 2001).

To date, many researches have been done to develop numerous RNA extraction protocol. Mostly rely on guanidine/ guanidinium salts (Chomczynski & Sacchi, 1987; Logemann *et al.*, 1987, cited in Liu *et al.*, 1998), sodium dodecyl bromide (SDS)/ phenol (Hosein, 2001) and CTAB-LiCl (Azevedo *et al.*, 2003; Zeng & Yang, 2002).

Due to the ineffectiveness of guanidinium salt solutions in isolate significant amounts of RNA from black spruce dry seed. Tai *et al.* (2004) had developed urea-LiCl method which uses urea instead of guanidinium salts as a denaturant. This method successfully isolated 5 to 10 µg of total RNA from 100 mg of black spruce dry seeds with the given $A_{260/280}$ ratio approximately 2.0. However, Kansal *et al.* (2008) pointed out that the method described by Tai *et al.* (2004) is time consuming regarding the time of centrifugation during separation and precipitation of RNA.

Furthermore, Singh *et al.* (2003) used the guanidine hydrochloride-based buffer system to isolate RNA from wheat and other carbohydrate-rich seeds. 148 µg of RNA was successfully isolate from 100 mg of tissue. This protocol just took 3 and a half hours for isolation RNA and able to obtain pure RNA which has the $A_{260/280}$ ratio of 1.85 (Singh *et al*., 2003). Whereas, Zeng and Yang (2002) mentioned that guanidine-based

method were unsuccessfully extracted RNA from high viscous samples rich in polyphenols and polysaccharides. The RNA pellet obtained from this method is brown and water insoluble (Zeng & Yang, 2002).

Moreover, Ding *et al.* (2008) developed an acid phenol-silica particle based method to isolate high-quality RNAs from various plant tissues recalcitrant to extraction. Using this protocol, they successfully isolated high quality of RNA with less contamination. Besides, RNA sample that obtained also showed intact bands in the agarose gels (Ding *et al.*, 2008). Sangha *et al.* (2010) expressed their view that although this protocol is a quick and effective method, preparation of silica particle is time consuming step that took about 24 h and the yield is low. Moreover, this method also has safety issues in handling and disposal since it required the use of toxic acid phenol for extracting RNA.

Besides, a method that combined the CTAB based RNA extraction method and a silica column of a commercial plant RNA extraction kit had been developed by Sangha *et al.* (2010) in order to isolate RNA from immature seeds of *J. curcas* L. This method was able to reduce the extraction time from two days to 3 hours by skipping the LiCl precipitation step. The ratio of A_{260}/A_{280} and A_{260}/A_{230} were greater than 2.0, which indicating that no contamination with protein and polysaccharides compound. The yield of total RNA obtained by using this method is 282.42 ± 12.91 µg/ g (Sangha *et al.*, 2010).

3.0 MATERIALS AND METHODS

3.1 Sample Collection

Early stage of *J. curcas* fruits, at 4 to 5 weeks after flowering was selected for total RNA isolation. Sample of Jatropha fruits were obtained from Alam Widuri Sdn. Bhd. For sampling purpose, early stage of Jatropha fruits was randomly collected from different trees. After obtained the seeds from the fruits, the seeds were immediately preserved in liquid nitrogen and stored at -80 ^{o}C until further use.

3.2 Preparation of Sample for Total RNA Isolation

Total RNA isolation was carried out by using three different protocols, namely, CTAB-LiCl method I, CTAB-LiCl method II and CTAB-isopropanol.

3.2.1 CTAB-LiCl Method I

Total RNA was extracted by using Sangha *et al.* (2010) and Zeng and Yang (2002) method with some modifications. Before grinding, the kernels of Jatropha seeds were separated from the seed shell. 0.5 g of Jatropha seeds sample was frozen in the liquid nitrogen rapidly and grind into a fine powder with a pestle and mortar. The frozen powder was transferred to a pre-chilled 50 ml Falcon tube. 5 ml prewarmed extraction buffer (Appendix A) was added to the sample and mix completely by inverting the tube. The sample was incubated for 30 minutes at 65 ^{o}C in a water bath with vigorous shaking several times.

After incubation, an equal volume of Chloroform: Isoamylalcohol (24:1) was added to the sample in a fume hood and vortexed for 30 seconds. Then, the sample was centrifuged at 6,000 rpm for 30 minutes at 4 ^{o}C. Thereafter, the aqueous supernatant (1

ml/ tube) was transferred into 2 ml RNase-free microcentrifuge tubes and re-extracted with an equal volume of Chloroform: Isoamylalcohol. Subsequently, it was centrifuged again at 10,000 rpm for 10 minutes at 4 ^{o}C. The supernatant was collected very slowly and carefully in order to avoid taking in chloroform. Then, it was centrifuged at 13,000 rpm for 30 minutes at 4 ^{o}C to pellet and discard the insoluble material. The supernatant was precipitated by addition of 0.25 volumes of 10 M LiCl (Appendix A). The mixture was mixed by inversion and the tube was standing for overnight at 4 ^{o}C to precipitate the RNA.

The next day, after precipitation of RNA, the RNA was pelleted by centrifugation at 13,000 rpm for 45 minutes at 4 ^{o}C. The supernatant was completely discarded. Then the pellet was washed 3 times with 70 % ethanol and centrifuged at 13,000 rpm for 2 minutes at 4 ^{o}C. Total RNA was air dried for 10 minutes and dissolved in DEPC-treated water (Appendix A). After that, total RNA was stored at -80 ^{o}C until use.

In this study, total RNA extraction method was repeated by modifying the concentration of LiCl (Table 3.1).

Table 3.1: RNA isolation with different LiCl concentration (M). RNA precipitation step was repeated with different molar concentration of LiCl in order to find out the optimum molar concentration of LiCl that able to precipitate RNA effectively.

Replication	I	II	III	IV
Molar concentration of LiCl (M)	8	9	10	11

3.2.2 CTAB-LiCl Method II

Total RNA was extracted by using Azevedo *et al.* (2003) method with some modifications. Before grinding, the kernels of Jatropha seeds were separated from the seed shell. 0.8 g of Jatropha seeds were grounded with mortar and pestle with liquid nitrogen into fine powder form.

The CTAB extraction buffer (10 ml) [Appendix A] was added inside a 50 ml falcon tube and preheated at 42 °C for 10 minutes. 0.8 g of frozen tissue was immediately added to the extraction buffer and mixed well by vortex. The sample was incubated for 90 minutes at 42 °C in a water bath with vortex several times. One volume of chloroform-isoamyl alcohol (24:1) was then added and mixed by vortex. The sample was then centrifuged at 6,000 rpm for 30 minutes at 4 °C. After that, the supernatant was transferred to another centrifuge tube and an equal volume of chloroform-isoamyl alcohol was added and centrifuge again at 13,000 rpm for 20 minutes at 4 °C. ¼ volume of 10 M LiCl (Appendix A) was added followed by transferred the supernatant into new 1.5 ml tube. The samples were then stored at -20 °C overnight. Next day, the samples were centrifuged 13,000 rpm for 15 minutes at 4 °C and the pellet was washed with 2 M LiCl (Appendix A) and centrifuged at 13,000 rpm for 15 minutes at 4 °C. Supernatant was then discarded and pellet was dissolved in 20 to 50 µl of DEPC- treated distllled water.

Starting from the LiCl precipitation step, the resulting supernatant was repeated by using differences LiCl concentration (Table 3.1).

3.2.3 CTAB-isopropanol

Total RNA was extracted by using Azevedo *et al.* (2003) method with replace the LiCl precipitation method with isopropanol. Before grinding, the kernels of Jatropha seeds were separated from the seed shell. 0.8 g of Jatropha seeds sample was grounded with mortar and pestle with liquid nitrogen into fine powder form.

The CTAB extraction buffer (10 ml) [Appendix A] was added inside a 50 ml falcon tube and preheated at 42 °C for 10 minutes. 0.8 g of frozen tissue was immediately added to the extraction buffer and mixed well by vortex. The sample was incubated for 90 minutes at 42 °C in a water bath with vortex several times. One volume of chloroform-isoamyl alcohol (24:1) was then added and mixed by vortex. The sample was then centrifuged at 6,000 rpm for 30 minutes at 4 °C. After that, the supernatant was transferred to another centrifuge tube and an equal volume of chloroform-isoamyl alcohol was added and centrifuge again at 13,000 rpm for 20 minutes at 4 °C. Equal volume of isopropanol was added followed by transferred the supernatant into new 1.5 ml tube. The samples were centrifuged at 13,000 rpm for 10 minutes at 4 °C. The pellet was washed with 70 % ethanol and stored at -20 °C overnight. Next day, the samples were centrifuge at 13,000 rpm for 10 minutes at 4 °C. Supernatant was then discarded and pellet was dissolved in 20 to 50 µl of DEPC-treated distilled water.

3.3 RNA Analysis by Using Agarose Gel Electrophoresis

After RNA isolation, size and integrity of isolated RNA were verified by running samples on 1% Agarose Gel Electrophoresis. 0.5 g of agarose was dissolved in 50 ml of 1 x tris-acetate (TAE) buffer in conical flask. After that, the solution was boiled in microwave for 1 minute. Agarose gel was then cooled down to between 55 °C to 65 °C. 1 µl of ethidium bromide was added and swirled to mix. After that, agarose gel was poured into the tray with comb. Agarose gel was then left for 30 minutes to solidify. After 30 minutes, TAE buffer was poured into the gel tank to submerge the gel to 2 to 5 mm depth. RNA sample (5 µl) was mixed with 1 µl of gel loading dye (bromophenol blue) on the parafilm by using pipette. Ladder (1 kb and 100 bp) and samples were loaded into the well and ran for 45 minutes at 100 V. After gel electrophoresis being successfully conducted, the ethidium bromide stained gel was visualized under UV transilluminator .

3.4 RNA Quantification

The quality and quantity of the total RNA extracts was quantified by using spectrophotometer. 5 µl of total RNA sample was diluted with 495 µl sterile distilled water in cuvette and quantified spectrophotometrically at wavelengths of 230 nm (A_{230}), 260 nm (A_{260}), 280 nm (A_{280}). The 260/280 and 260/230 absorption ratio was verified as quality indexes.

4.0 RESULT AND DISCUSSION

4.1 Preparation of Sample for RNA Isolation

In this study, around 40 seed samples were used because isolation of total RNA from Jatropha seeds are difficult due to its high quantity of polysaccharides, carbohydrates, lipids, polyphenols and proteins. After early stage of Jatropha fruits were collected from Alam Widuri Sdn. Bhd., Jatropha fruits were cracked to open and seeds were obtained. Jatropha seeds were stored at -20 °C but this is not an effective storage method. This is because these seeds will be rotted after two or three weeks. Thus, seeds were better ground into fine powder with liquid nitrogen and then stored at - 80 °C. This storage method can keep the sample for long term use.

4.2 Isolation of Total RNA

In this project, total RNA was isolated from early stage of Jatropha seeds and followed by the analysis of total RNA quality and quantity.

4.2.1 CTAB-LiCl Method I

There was 0.5 g of Jatropha seed samples in the powder form used to isolate total RNA. Two 28 S rRNA (between 400 bp and 500 bp) and 18 S rRNA (about 300 bp) bands were observed from gel electrophoresis result (Figure 4.1). However, these two bands were slightly visible with smearing along the lanes and the 28S band was approximately twice the integrity of the 18S band. This indicated that total RNA was contaminated and somewhat degradation had occurred.