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Original Article

Optimization of High-Yielding Protocol for Dna Extraction From-Leaves of Asam Gelugor (*Garcinia Atroviridis*), Malaysian High Economic Value Of Medicinal Plant

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Abstract *- DNA isolation is difficult in woody plants due to polysaccharides, tannins, alkaloids, polyphenols, and other secondary metabolites that interfere with isolated DNA quality. Here we report for the first time a fast, reliable, and less expensive method to isolate genomic DNA from leaves of Asam Gelugor (Garcinia atroviridis). This method generally started with a sample washing step by Triton buffer (2%) before isolating gDNA according to a modified CTAB method. We employed a high concentration 4% (w/v) CTAB, 1.5% (w/v) PVP (Polyvinylpyrrolidone), 0.3% (v/v) mercaptoethanol and relatively higher concentration of 30 mM EDTA compared to extraction method developed for other Garcinia varieties. The method has included a precipitation step by using 5M NaCl and chilled ethanol to increase the solubility of polysaccharides and 50 mM ascorbic acid to inhibit polyphenol oxidase activity. High purity of gDNA was obtained as evidenced by the A260/A280 ratio ranging from 1.7 to 1.8, which suggested that proteins and RNA did not contaminate the purified gDNA. DNA concentration ranged from 1.5 to 5.0 µg/µl. This newly modified protocol enabled the isolation of high-quality Garcinia atroviridis genomic DNA for subsequent genetic diversity study of germplasms and gender identification.*

Keywords *- Garcinia Atroviridis, DNA extraction, Optimization, Leaf tissue.*

1. Introduction

In Malaysia, *G. atroviridis*, locally known as Asam Gelugor, is not much exploited compared to G. mangostana, whose fruit is delicious and well commercialized. In recent times, Asam Gelugor has contained an anti-inflammatory and antioxidant compound named (-) hydroxyl citric acid (HCA) that makes this fruit medicinal importance and, at the same time, potential for commercial via industrial products [1]. Asam Gelugor is among tree species with a gender differentiation problem that can be solved between 7-8 years after the plant starts to bear the flower [2]. Genotyping and markers technology can help to determine the sex of the plant at the juvenile stage. DNA must be pure, intact, and high-quality [3].

Genomic DNA isolated from plants is normally compromised by excessive contamination of secondary metabolites [13]. Thus, depending on the plant species, the DNA extraction protocol needs to be optimized due to the presence of these metabolites [27]. Searching for an efficient protocol to extract gDNA from plants with a high level of secondary metabolites led us to develop a protocol for obtaining higher quality and yield DNA [4-7].

The ultimate aim of extracting genomic DNA is to obtain undigested nuclear material. The endonuclease is one of the important problems, especially in isolating/purification of high molecular weight DNA, which its reaction can cause degradation [8]. Inhibition of enzymatic activity by Polysaccharides had been proved by their interruption with *Taq* polymerase and Restriction of enzyme activity [10-11]. Existing polysaccharides in DNA samples may be particularly problematic, and their presence is characterized by the formation of a highly viscous solution [9]. Also, oxidized polyphenols released by crushed plant cells will covalently bind to DNA and give a brown color to the extracted DNA, making it useless for molecular studies [12].

An ideal extraction technique includes optimizing DNA yield, minimizing DNA degradation, and being efficient in terms of cost, time, labor, and supplies. Besides

that, the optimized protocol can be applied for extracting multiple samples and generating minimal hazardous waste. The present study develops to fulfill these issues by isolating the total genomic DNA from young leaves of *Garcinia atroviridis*. The optimized protocol involves washing the ground sample before extracting the DNA by utilizing various percentages of CTAB buffer to obtain free contamination of high-quality gDNA.

2. Materials and Methods

2.1. Plant Material and Sample fixation

Fresh, young pinkish, and healthy leaves of *G. atroviridis* were randomly sampled from the plant grown in Biotechnology and Nanotechnology Research Centre, MARDI, Malaysia (Figure 1) and immediately frozen in liquid nitrogen.

The samples were fixed by grinding to a fine powder in the presence of liquid nitrogen using mortar and pestle and stored at -80°C until its ready to be DNA extracted.

Fig. 1. Fresh, pinkish in color, and healthy leaves of *G. atroviridis* used in this study to extract high-yielding of genomic DNA.

2.2. DNA Isolation Methods

Plant gDNA extraction kit (QIAGEN, USA), a slightly modified CTAB method with 3% CTAB (according to [14]), and SDS-based DNA extraction methods [15] were utilized to extract gDNA from *G. atroviridis* leaf samples. However, none of these methods were given the interest DNA band on the agarose gel. Since the CTAB DNA isolation method developed by [16] to extract genomic DNA from mature trees yielded convincing results, this method was optimized to extract high-quality gDNA from *G. atroviridis* leaf sample by varying the concentration of CTAB, Tris-HCl, NaCl (Sodium Chloride), β-Mercaptoethanol, and PVP (Polyvinylpyrrolidone).

2.3. Optimized Extraction Method

The optimized protocol starts with the washing step by applying 2% Triton to the ground sample. This step would remove polysaccharides and phenolic compounds attached to the DNA. Extraction buffer containing 4% CTAB was found to be an optimized percentage of CTAB that allowed a great improvement in both DNA yield and purity. The optimized procedure was described as follows.

2.4. Pre-extraction Washing

Place approximately 3g of ground sample in a 50ml centrifuge tube and add 30ml of freshly prepared Triton buffer (2% Triton X-100, 0.3M NaCl, 0.1M
Tris Buffer, and 0.03M EDTA). Gently swirl the tubes a few times and centrifuge at 8000 rpm, 15 min at 4°C. Carefully discard the highly viscous supernatant to minimize wasting the pellet. This Triton washing step can be repeated 2-3 times using older leaves samples (dark green).

2.5. DNA isolation by modified CTAB method

i. Preheat freshly prepared extraction buffer containing 4% CTAB (w/v), 100mM Tris-HCl (pH7.5), 30mM EDTA, 1.4M NaCl, 50mM Ascorbic acid, 1.5% PVP (w/v) (M.V. 40,000) and 0.3% Mercaptoethanol (v/v) (freshly added just before use) in water bath at 60°C.
ii. Add 9ml CTAB extraction buffer (3ml/1g sample) to the centrifuge tube containing the washed sample and thoroughly homogenize the tissue.
iii. Incubate the sample in the water bath at 60°C for 1 hour between shaking.
iv. Allow the tube to cool before adding an equal amount of chloroform: isoamyl alcohol (24:1); mix well by inversion.

v. Centrifuge the solution mixture at 8,000 rpm for 25 min at 4°C.
vi. Collect the aqueous layer and add an equal amount of chloroform: isoamyl alcohol (24:1), mix the solution gently and centrifuge at 8,000 rpm for 15 min at 4°C.
vii. Collect the aqueous layer and repeat step (vi) to keep the purity of the DNA sample.
viii. Collect the aqueous/supernatant into a new tube, adding 0.5 volumes of 5M NaCl and 2 volumes of chilled ethanol.
ix. Allow the DNA to be precipitated at -20°C for at least 1 hour.
x. Centrifuge the tube at 5000 rpm, 10 min at 4°C to harvest the DNA pellet.
xi. Add 1.5ml chilled 80%/75% ethanol into the tube to wash the pellet. Recover the pellet by centrifuge at 5000 rpm, 5 min at 4°C.
xii. Throw the supernatant and allow the pellet to dry in a fume hood.
xiii. Add 100µl SIGMA water to dissolve the pellet
xiv. RNase Treatment by adding 10µl of 10mg/mL RNase stock solution per 50µl sample and incubating at 37°C for about 4 hours.
xv. Store purified gDNA at -20°C until used.

The steps in this protocol for isolating gDNA from *G. atroviridis* are illustrated in a flow chart, as shown in Figure 2.

2.6. Quality of gDNA by Qualitative and Quantitative Analysis

The DNA yield (purity and concentration) was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) at 260 nm. The purity of extracted DNA was obtained by calculating the absorbance ratio A260/280. Further DNA purity assessment was electrophoresis of all DNA samples in 1% agarose gel, stained with 0.25 µg/ml ethidium bromide, and visualized/photographed using a gel documentation system (Biorad, USA).

2.7. Downstream Application of extracted gDNA.

2.7.1. Restriction Digestion

Extracted gDNA was subjected to digestion by restriction enzymes. The reaction mixture containing 5µl DNA samples, 1µl of 10X assay buffer, and 2 µl of each restriction enzyme (EcoR1 and HindIII) was prepared and incubated at 37°C for 1 hour to complete the digestion. Digested products were assessed by electrophoresis onto 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

2.7.2. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR)

Three random primers for each RAPD and ISSR were obtained from the public database and synthesized by IDT (Integrated DNA Technologies Inc.) (Table 1). The PCR was carried out in a total of 25 µL reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 2 µM primer, and 0.5 U of Taq DNA polymerase (Promega Corp., USA), and 50 ng of template DNA. The amplification was performed in a thermal cycler (BioRad DNA Engine Tetrad 2 Peltier) through initial denaturation for 5 min at 94°C, 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 53°C, and elongation for 1 min 30 sec at 72°C followed by final extension for 10 min a 72°C. The amplified products were observed in gel documentation (BioRad Gel Doc. XR) upon 1.0% agarose gel electrophoresis.

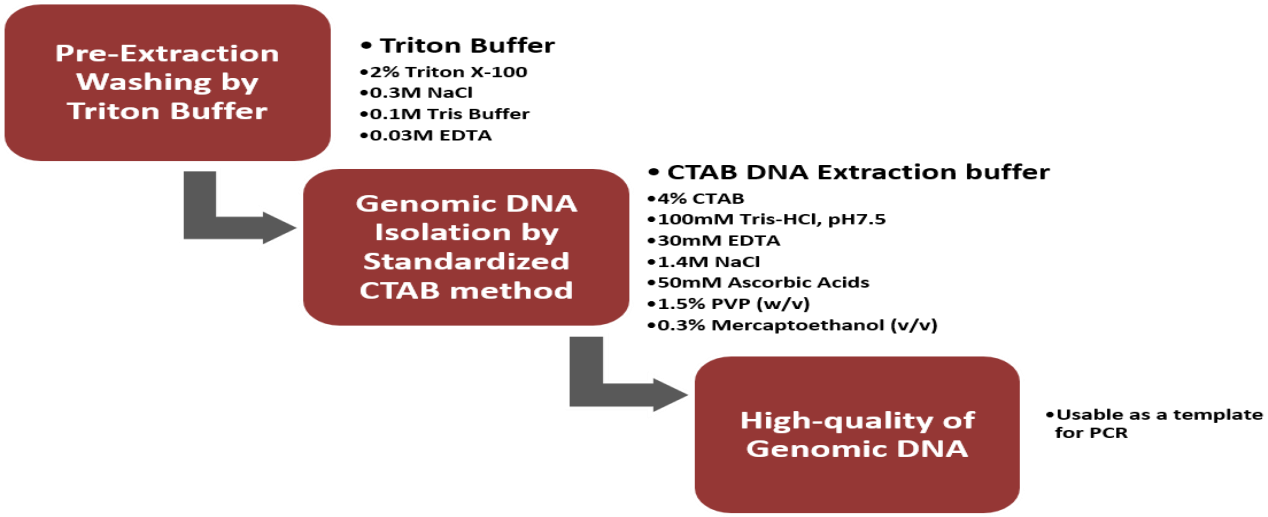

Fig. 2 Flow chart illustrates the gDNA isolation and purification protocol from *G. atroviridis* leaf.

2.7.3. Amplification of DNA barcoding gene, matK

The gene was amplified in a 25 µl PCR reaction containing 1X PCR buffer, 2.5 mM $MgCl_2$, 1 mM dNTPs mix, 20 mM of each universal primers for matK gene (Table 1), 50ng of template DNA, and 1.0 U Taq DNA polymerase. Amplification was carried out by using a one-step touchdown PCR the program, which consists of 2 cycles of PCR. The first cycle was at 96°C for 90 sec, 50°C for 60 sec, and 68°C for 2 min and followed by 35 cycles for 30 sec at 95°C, 2 min at 68°C for the second cycle. Another 20 min at 68°C was added for final elongation. 1% agarose gel buffered with TAE was used to separate the PCR products. The gel was stained with ethidium bromide to observe the bands via a gel documentation system (BioRad Gel Doc. XR.

Table 1. List of primers utilized to show the efficiency of PCR by using the gDNA obtained from this present study

Primer name	Primer sequence 5'- 3'(length)
RAPD-OPA-13	CAGCACCCAC
ISSR-10	GC + $(AG)_7$
matK (trnk-F)	GGGTTGCTAACTCAATGGTAGAG
matK (trnK-R)	TGGGTTGCCCGGGGCCGAAC

3. Results and Discussion

Obtaining high-quality DNA from woody/mature tree species is an important issue if the DNA sample needs to be used in molecular works [21-23]. Tissues from this tree often contain polysaccharides and phenolic compounds in large amounts, becoming a major problem separating gDNA from it. Even if the gDNA extraction is succeeded, these cellular components can still contaminate extracted DNA. Hence, removing polysaccharides and phenolic compounds is critical before extracting the DNA, as contamination by these compounds can inhibit downstream molecular reactions [19-20].

An important aspect to be taken toward extracting high-quality gDNA is the size and age of the leaf sample. Hence, fresh and young leaf materials that are pinkish were suitable for gDNA isolation (Figure 1), and mature leaves were avoided due to higher quantities of polyphenols and polysaccharides [4]. Subsequently, the gDNA isolation method uses Cetyl Trimethyl Ammonium Bromide (CTAB) as the main component in the extraction buffer developed by [14] had been modified in this study by considering standardization at a varying concentration of Tris-HCL, β-mercaptoethanol, NaCl, and PVP (Figure 3).

Washing steps were added to the protocol recommended by [16] when dealing with woody/mature tree samples. High quantity/quality of purified gDNA obtained once the Triton washing step was included in the protocol. This proved that Triton washing step is very important, and by omitting this step, gDNA failed to be extracted. A high level of secondary metabolites in the *G. atroviridis* leaf contributed to this issue. This compound is not easily separated from the DNA during the extraction process. Triton is a highly viscous nonionic detergent and very popular in extraction protocol because it removes most plant secondary metabolites. Hence, incorporating a Triton washing step proves to be essential for producing consistent results.

CTAB is a detergent to break up the lipid-associated membrane by solubilizing the membrane before forming a complex to release DNA. The concentration of CTAB would be altered to increase the capability of this detergent to remove proteins from the extracted DNA [26]. In addition, the optimum concentration of PVP with high molecular weight (40,000) attributes to obtaining high-quality DNA by less tendency to precipitate with the nucleic acids. The efficiency of PVP is increased by working along with the high molar concentration of NaCl to inhibit co-precipitation of polyphenols, polysaccharides, and DNA from yielding a sufficient amount of polyphenol-free DNA [24]. Hence, to obtain high-quality gDNA, we found that 4% CTAB, 1.5% PVP, and 1.4M NaCl is the optimal combination for the extraction buffer.

Exposing released polyphenols to the atmospheric oxygen will form tannins and melanins via oxidation. These compounds have a high affinity to covalently bind with DNA and coprecipitate once alcohol is added to form a highly viscous solution brown in color [25]. In this present study, ascorbic acid (50mM) as an antioxidant was added to change the pH of the extraction buffer. Hence, the action of polyphenol oxidase would be inactivated to prevent the oxidation of polyphenols and subsequently improve the quality and quantity of extracted DNA to be used for restriction digestion and molecular fingerprinting. β-mercaptoethanol is a strong reducing agent, and the contribution of this compound in DNA extraction protocol has been proved to remove the polyphenol during the extraction process [17]. Therefore, adding 0.3% of β-mercaptoethanol makes the protocol excellent for extracting high-quality DNA from *G. atroviridis* leaf samples. No visible DNA fragmentation in any samples due to shearing DNA upon extraction. Reproducible results and the absence of smears further strongly support the efficiency of this method in extracting high purity of gDNA.

The ratio of A260/A280 ranges from 1.7 to 1.8, and more than 2 for the ratio of A260/A280 shows excellent purity of extracted DNA. This suggests that the modified methods can produce the sufficiently free gDNA of proteins and polyphenolics/polysaccharide compounds [18]. In addition, no RNA contamination was detected by agarose gel electrophoresis (Figure 3). Clear banding patterns upon amplification of gDNA by RAPD and ISSR (Figure 4) together with amplification of barcoding gene, matK (by universal primers) (Figure 5) show that shearing of gDNA by this modified protocol did not interfere with the enzymatic reactions as well as no inhibition of *Taq* DNA polymerase activity. Hence, this modified protocol's quality of extracted gDNA is suitable for downstream applications in molecular phylogenetic studies of G. atroviridis. The protocol could also be useful for extracting high-quality gDNA from other plant species with high polysaccharides and secondary metabolites.

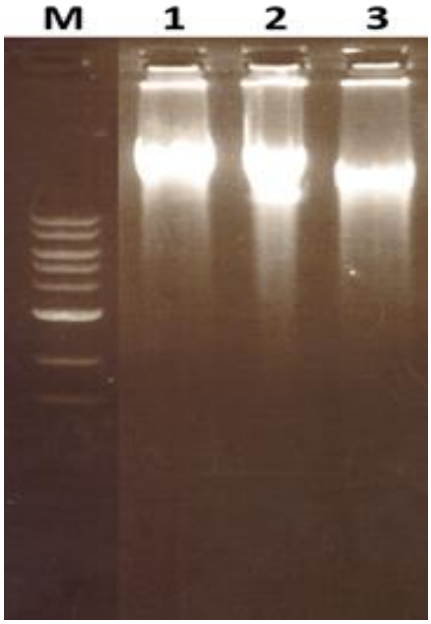

Fig. 3 Genomic DNA isolated from leaf samples by the present optimized extraction methods and resolved under 1% agarose. The samples were isolated from fresh young leaves of *Garcinia atroviridis* collected from 3 genotypes of the plant as represented by lanes 1- 3.

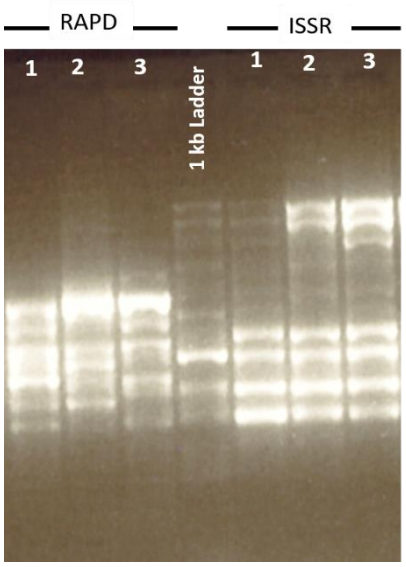

Fig. 4 RAPD (OPA-13) and ISSR (ISSR-10) primers banding pattern of the three *Garcinia atroviridis* genotypes.

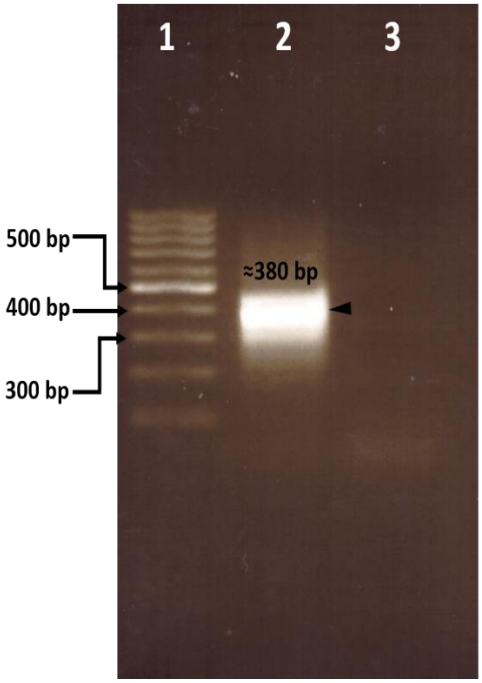

Fig. 5 Amplification of *Garcinia atroviridis* matK gene. Lane 1: 100bp DNA ladder; Lane 2; matK gene amplicon, which is about 380bp; Lane 3: Negative control (water)

5. Conclusion

Due to high levels of secondary metabolites and polysaccharides, existing methods need to be modified to extract high-quality gDNA from *G. atroviridis* leaves. High-quality gDNA is extracted from *G. atroviridis* leaf using this modified method. The extracted gDNA fulfills the PCR-usable standard proved by its capability to be amplified for RAPD, ISSR, and barcode genes. Besides yielding high-quality gDNA, the protocol developed in this study is robust and efficient to make the protocol possible for whole-genome sequencing of *Garcinia* species for advanced bioinformatics investigations. Hence, this modification method developed in the study is a marked improvement over the earlier reported methods to extract gDNA from *Garcinia* species, which varies in terms of secondary metabolites and polysaccharides.

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