Short Communication

Improved protocol for isolation of genomic DNA from leaf tissues of *Phyllanthus emblica* Gaertn

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Abstract
Modified Cetyltrimethylammonium bromide (CTAB) protocol for DNA isolation was developed from leaf tissues of *Phyllanthus emblica* for obtaining high quality genomic DNA. Fresh leaves of three different maturity were analyzed for yield and quality of DNA. Acidity was determined in three different maturity of leaves viz. tender, intermediate and mature and their influence on DNA quality was determined. Drastic reduction of pH was the primary cause for poor quality of DNA. However, high quality DNA isolation was achieved by stabilizing the pH by addition of NaOH during different stages of DNA isolation process. The present protocol yielding high quality intact DNA for genetic fingerprinting as well as for amplification of chloroplast genes for molecular analysis.

***Keywords*:** Phyllanthus emblica; chloroplast DNA; restriction enzymes; trnL (UAA) intron sequences; gene specific primers

Phyllanthus emblica, popularly known as Indian gooseberry is one of the important medicinal and vitamin C enriched fruits used for various formulations of medicine and food products. It grows abundantly in the forests of India and many South Asian countries (Pathak *et al*., 2003; Singh, 2003). A large number of heterogeneous populations with wide range of genetic variations due to cross pollination has widened the scope of natural selection and genetic improvement of *P. emblica* by conventional as well as by molecular breeding approaches. *P. emblica* being a perennial crop, molecular tools can be of great utility to support the conventional breeding. Therefore, isolation of intact high quality DNA is essential for carrying out molecular studies. Although, a number of protocols have been developed for purifying the DNA from phenols, polysaccharides and secondary metabolites, degradation of genomic DNA is one of the major constraints in many plant species, leading to improper or no amplification of DNA in PCR based studies. Problems encountered in the isolation of high molecular weight DNA from several of the medicinal and aromatic plant species has been attributed to degradation of DNA due to endonucleases activities, co-isolation of viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions (Weishing *et al*., 1995). Presence of polyphenols, which is powerful oxidizing agents present in many plant species reduce the yield and quality of DNA (Porebski *et al*., 1997).

A number of protocols have been described for isolation of high quality DNA from various plant species (Deshmukh *et al*., 2007; Chakraborti *et al*., 2006; Fu *et al*., 2004; Aras *et al*., 2003; Mace *et al*., 2003; Sharma *et al*., 2003). However, there is no satisfactory protocol could be developed for isolation of high qual-

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ity DNA from leaf tissues of *P. emblica*, primarily due to high acidic nature as it contains highest amount of vitamin C as compared to any other plant species. DNA isolation from leaf tissues of *P. emblica* have been described and reported that reduction of pH to extreme acidic level during DNA isolation process was one of the major factors responsible for degradation of DNA (Warude *et al*., 2003). However, the problem in obtaining high quality DNA remains unsolved. We report a reliable and effective protocol for isolation of high quality genomic DNA from leaf tissues of *P. emblica* without degradation of DNA by stabilizing the pH of buffer with NaOH at different stages in the process of DNA isolation. Further, we have also demonstrated that the DNA isolated using the present protocol without employing liquid nitrogen can be used for DNA fingerprinting and chloroplast genome analysis of various germplasm of *P. emblica*.

Trees of *P. emblica* maintained in the botanical garden of Sri Paramakalyani Centre for Environmental Sciences, Manonmaniam Sundaranar University become the source of leaf tissue for the present study. Healthy uninfected leaf samples attained at three different maturity levels viz. a) Immature tender leaves, b) Fully expanded young leaves and c) mature leaves were collected and thoroughly washed with sterile water before grinding of samples under extraction buffer.

One gram of leaves were cut into tiny pieces and ground thoroughly using prechilled mortar and pestle without liquid nitrogen under extraction buffer (Tris HCl 100 mM, 0.5 M EDTA, 1.5 M NaCl, 2% CTAB). β-mercaptoethanol (300 µl v/v) was added during the grinding of samples. Samples were incubated at 65ºC for 60 min with gentle mixing of samples at a regular time intervals. They were allowed to attain room temperature (RT) and equal volume of chloroform/isoamyl alcohol (24:1) was added with gentle mixing before spinning the samples at 8000 rpm for 10 min. Clear supernatant of the samples were recovered and 2/3 volume of isopropanol and 500 µl of 0.5 M NaCl was added before incubation at -20ºC over night. Precipitated DNA after spinning of samples at 8000 rpm for 10 min were recovered by discarding the supernatants and pellets were washed with ethanol (70%). Pellets were allowed to dry at RT and dissolved in 200 µl of TE (Tris 10 mM and EDTA 1 mM) buffer. Samples were treated with RNase before determining the quality and quantity. In the process of isolation of genomic DNA from the leaf tissues of three different maturity levels, the pH of the sample was carefully checked at two stages (1, during grinding of leaf tissues along with DNA extraction buffer and 2, at different time intervals during incubation of samples at 65ºC) using a small handy pH meter and the change in pH at different time intervals were recorded and analyzed in normal protocol described above. In the modified protocol, NaOH (0.1N) was added at two stages (500 µl during grinding of leaf tissues and 20 µl at every 15 min time intervals during incubation at 65ºC) and the stability of pH was recorded. Other steps followed for isolation of DNA was similar to that of the above procedure.

The yield of DNA per gram of leaf tissue was determined using UV visible spectrophotometer at λ260. The purity of DNA was determined by calculating the ratio of absorbance at λ260/λ280. Concentration and purity of DNA from various samples was further determined by running the DNA samples on 0.8% agarose gel and compared with λDNA. The suitability of isolated DNA for molecular analysis was verified by digesting the genomic DNA with *Eco*RI. Digestion of DNA was carried out in 20 µl volumes containing 4 µl of DNA, 2 µl of 10 X assay buffer, 2 µl of BSA (10 mg/ml) and 8 units of *Eco* RI at 37ºC overnight. The digested DNA was subjected to electrophoresis using 1.0% agarose gel and visualized using Gel Documentation System (Lark Innovative Teknowledge, India). The present protocol was extended for isolating DNA from different commercial varieties of *P. emblica* and qualitative and quantitative assessment of DNA was carried out.

For amplification of genomic DNA using random primers, 25 µl of reaction mixture containing 25 ng of genomic DNA, 0.6U of Taq DNA polymerase, 100 µl of each dNTP, 1 X Taq DNA polymerase buffer and 10 pmol of operon random primer (OPA 4) was used and amplification was performed using Thermal Cycler (Eppendorf) with the following PCR conditions: 94ºC for 5min; 40 cycles of 94ºC for 1 min, 36ºC for 1 min and 72ºC for 2 min and final extension at 72ºC for 5 min. PCR products were subjected to agarose gel (1.5%) electrophoresis in 0.5 X TBE buffer along with DNA ladder (Helini Biomolecule, Chennai, India) and observed for ensuring the suitability of genomic DNA for PCR amplification. PCR amplification of chloroplast *trn*L (UAA) intron was performed using the universal chloroplast gene specific primers 5´CGAAATCGGTAGACGCTACG3´ (A49325) and 5GGGGATAGAGGGACTTGAAC3 (A49865) as this gene is reported to be one of the widely used candidate genes for DNA barcoding and molecular taxonomy

(Taberlet *et al*., 1991). The PCR reaction mixture consisting of master mix 25 µl (contains: 10 X Taq buffer, 2 mM $MgCl_2$, 0.4 mM dNTP mix, and 2U proofreading Taq DNA polymerase) procured from Helini Biomolecules, Chennai, (India), 10 pmoles of each forward and reverse primers, template DNA (2 ng) and nuclease free water (21 µl) to make final volume to 50 µl. The PCR reactions were 94ºC for 3min (Initial Denaturation), 30 cycles of 94ºC for 1 min (Denaturation), 50ºC for 1min (annealing): 72ºC for 1 min (Extension) and 72ºC for 5 min (Final extension). Amplified product was subjected to DNA sequencing using ABI 373 automated sequencer (Applied Biosystems, Inc.) using a DyeDeoxyTM Terminator Cycle Sequencing kit (Applied Biosystems) as recommended by the manufacturer and analyzed using the BLAST and submitted to NCBI.

Periodic observation of leaf extracts in three different maturity levels of leaf samples during isolation of genomic DNA revealed a sharp reduction of pH from 8-4.2 when standard CTAB method of DNA isolation was followed. However, the pH was more or less stable from 7.41-7.66 in three different maturity levels of leaf extracts when NaOH (0.1 N) was added during grinding and incubation of samples at 65ºC (Table 1). This experiment demonstrated the extent of reduction in pH at different time intervals and also leads to optimization of protocol for maintaining the requisite pH to overcome DNA degradation due to lowering of pH. Qualitative and quantitative analysis of DNA in three different maturity levels of leaf samples by spectrophotometric method revealed that DNA extracted using normal protocol yielded poor quality DNA with significant reduction of yield. In contrast, DNA isolated using the modified protocol by stabilizing the pH through addition of NaOH yielded high quality DNA and λ260/λ280 ratios of these samples were ranged from 1.92 to 2.03. Increase in λ260/λ280 ratio to 2.03 in one of the samples is possibly due to RNA contamination. However, this can be overcome by treating the DNA with RNAse. (Table 2). DNA isolated using normal protocol when subjected to agarose gel electrophoresis, degradation of DNA was commonly noticed in all the samples (Fig.1A). However, modified protocol yielded intact genomic DNA of high quality with little or no degradation (Fig. 1B).

Quantity of DNA was increased nearly 3-5 times depending upon the maturity of leaf samples. Our study confirmed that young leaves are more suitable for high yield of DNA than tender and mature leaves. DNA isolated by employing the modified protocol could be effectively digested with *Eco*RI as revealed by agarose gel electrophoresis and possible contaminants like proteins and carbohydrate were not interfered the mobility of digested DNA (Fig. 2A). DNA samples isolated by employing the modified protocol from three different maturity of leaves did not show marked differences with respect to quality but differed in their yield of DNA. Isolation of genomic DNA from young leaf tissues of different commercial varieties of *P. emblica* (Banarasi, NA-9, Krishna, Chakaiya and Kanchan) by employing the modified protocol yielded reproducible results and high quality of genomic DNA were obtained. The λ260/λ280 ratios of these samples were ranged from 1.74-1.95 revealing the acceptable quality of DNA for PCR based molecular studies. All

Table 1. Changes in pH of leaf extracts of *P. emblica* in normal (without stabilizing pH) and improved (stabilizing pH with NaOH) protocol of DNA isolation.

Type of leaf sample	Reduction of pH during Incubation at 65°C (min)				
	0	15	30	45	60
Without stabilizing pH (Normal protocol)					
Immature tender leaves	6.87± 0.14	6.52± 0.16	6.38± 0.13	4.78 ± 0.11	4.32± 0.10
Fully expanded young leaves	6.62± 0.17	6.42± 0.11	6.16± 0.10	5.75 ± 0.39	4.20± 0.24
Hard and mature leaves	6.72± 0.14	6.42± 0.19	5.96± 0.33	4.87 ± 0.23	4.33± 0.15
Stabilizing pH with NaOH (0.1N) (Improved protocol)					
Immature tender leaves	7.95± 0.02	7.87± 0.02	7.65 ± 0.03	7.42 ± 0.02	7.66± 0.02
Fully expanded young leaves	7.85± 0.01	7.66 ± 0.01	7.66 ± 0.02	7.69 ± 0.02	7.42± 0.01
Hard and mature leaves	7.84± 0.01	7.68± 0.02	7.65 ± 0.01	7.65 ± 0.02	7.41± 0.01

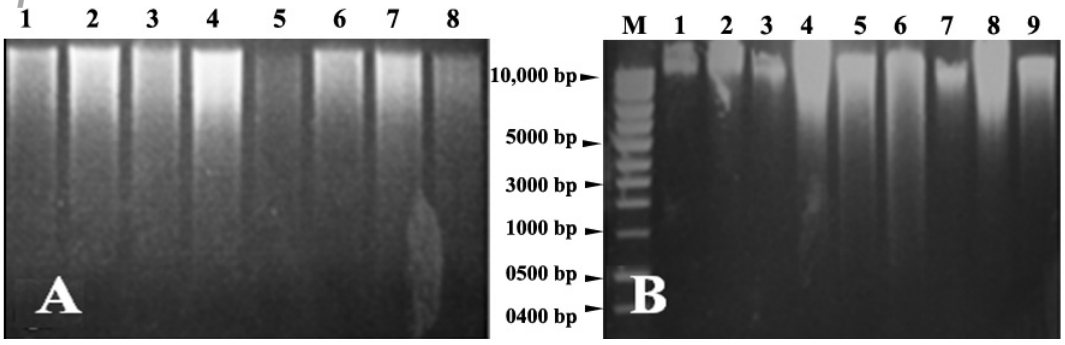

Figure 1. A: Degradation of genomic DNA due to acidic nature of tissue when different maturity levels of leaves namely immature (1-3), fully expanded young (4-6) and mature (7, 8) were used for DNA isolation. B: Improved method of DNA isolation by stabilizing the pH using NaOH during DNA isolation. M- High molecular weight DNA marker, Intact DNA of tender (1-3), young (4-6) and mature (7-9) leaves.

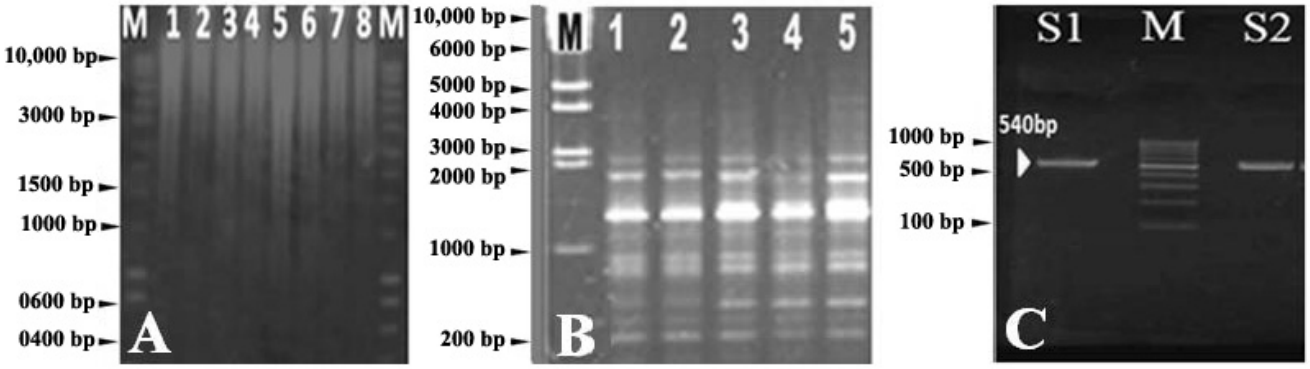

Figure 2. A: Digestion of genomic DNA of different leaf samples namely immature tender (1-3), fully expanded young (4-6) and mature (7, 8) with *Eco*R1. B: Amplification of genomic DNA of five popular varieties Banarasi (1), NA-9 (2), Krishna (3), Chakaiya (4) and Kanchan (5) of *Indian* Gooseberries using operon random primer (OPA-4), C: Amplified fragment of chloroplast *trn*L (UAA) intron S1- *P. emblica*, S2- *P. indofischeri* and M -100 bp DNA ladder.

the varieties of *P. emblica* yielded DNA ranging from 364-440 µg/g of leaf tissue (Table 3).

Genomic DNA isolated following the modified protocol when amplified with one of the random primers (OPA 4) to verify the suitability of DNA for genetic finger printing, around 8-10 sharp DNA bands were resolved in the agarose gel, revealing the suitability of genomic DNA for molecular characterization of Indian gooseberries (Fig. 2B). As these DNA fragments were very sharp ranging from 200-2320 bp, the present protocol is efficient for amplification of even larger genes around 3000bp without any degradation. When genomic DNA of two species of Indian gooseberries namely *P. emblica* and *P. indofischeri* were further tested for amplification of *trn*L (UAA) intron of chloroplast genome, PCR product of *trn*L (UAA) intron with the molecular weight of 540 bp was obtained (Fig. 2C) and these products were found suitable for DNA sequencing. Sequence data of *trn*L (UAA) introns corresponding to *P. emblica* and *P. indofischeri* revealed that the entire fragment of *trn*L (UAA) introns of both the species were intact without degradation of any base pairs, revealing the quality of chloroplast DNA obtained using the modified DNA isolation protocol. Sequencing of *trn*L (UAA) introns of *P. emblica* and *P. indofischeri* revealed very close resemblances with high degree of homology along with an another closely related species namely *Phyllanthus amarus* (Sangeetha *et al*., 2010). The *trn*L (UAA) introns sequenced from the present study was submitted to NCBI (Acc. No. FJ847837 for *P. emblica* and Acc. No. GU930706 for *P. indofischeri*). Thus, the present work demonstrated that our protocol is efficient enough to isolate nuclear as well as chloroplast

Table 2. Comparison of improved protocol for quality and quantity of DNA isolated from three different maturity levels of *P. emblica* leaves.

Type of leaf sample	λ260	λ280	λ260/ λ280	DNA (μg/μl)	DNA (μg/g)
Tender leaves					
1) Without stabilizing pH	0.0258	0.0198	1.303	1.29	129
2) With stabilizing pH	0.0843	0.0439	1.920	4.21	421
Young leaves					
1).Without stabilizing pH	0.0195	0.0125	1.560	0.97	097
2) With stabilizing pH	0.0892	0.0439	2.031	4.40	440
Mature leaves					
1).Without stabilizing pH	0.0125	0.0085	1.470	0.62	062
2).With stabilizing pH	0.0699	0.0359	1.947	3.49	349

Table 3. Quality and quantity of DNA obtained using modified protocol in different commercial varieties of *P. emblica* cultivated in India.

Varieties	Parentage	λ260	λ280	λ260/ λ280	DNA yield (μ/μl)	DNA yield (μg/g tissue)
1) Banarasi	Seedling selection of progeny in Varanasi open pollinated	0.0869	0.0449	1.930	4.345	434
2) NA-9	Open pollinated seedling progeny of Banarasi	0.0728	0.0395	1.843	3.640	364
3) Krishna	A chance seedling derived from Banarasi	0.0735	0.0421	1.745	3.675	367
4) Chakaiya	Seedling selection from wild Germplasm	0.0825	0.0421	1.959	4.125	412
5) Kanchan	Seedling derived from Chakaiya	0.0755	0.0398	1.896	3.775	377

genome for PCR based studies.

Many of the plant tissues, especially woody plants contain high level of tannins and other polyphenolic compounds that contribute for extreme acidic nature of tissue extracts during DNA isolation (Peterson *et al*., 1997). Method has been described for isolation of genomic DNA from frozen leaf tissues of *P. emblica* (Warude *et al*., 2003) with a minor modification of DNA isolation protocol described earlier (Murray and Thompson, 1980; Doyle and Doyle, 1987). They monitored each step of the protocol by precipitating the DNA followed by electrophoresis to determine the extent of DNA degradation. Though, DNA of amplifiable quality could be obtained by increasing the ratio of tissue to buffer for adjusting the pH 7.5 to 8, this protocol still relay upon on liquid nitrogen. In addition, the method described earlier (Warude *et al*., 2003) for isolation of genomic DNA by increasing the ratio of tissue to buffer may often interferes with DNA quality since the volume of tissue sample is higher than the

extraction buffer. Our protocol describes the simple method to stabilize around pH8 using NaOH rather than adjusting the pH by increasing the ratio of tissue to buffer as described in the previous report (Warude *et al*., 2003).

Isolation of genomic DNA from high acidic samples is extremely difficult since pH at extreme acidic conditions reduce the quality of DNA by degradation. It was reported that the original pH of the extraction buffer rapidly decreases due to release of endonucleases, polyphenols and several other secondary metabolites which are highly acidic in nature (Sharma *et al*., 2010). This problem has been reported in several plant species such as *Terminalia belerica*, *T. chebula*, including *P. emblica* (Sharma *et al*., 2010; Warude *et al*., 2003). Freeze drying of biological samples by lyophilisation or liquid nitrogen ensures isolation of high quality DNA (Agwanda *et al*., 1997). However, availability of liquid nitrogen is one of the major constraints in many developing countries, where the research centres are situated in very interior provinces. Thus the present protocol is very effective in isolation of high quality DNA without employing liquid nitrogen. Further, the problem of DNA degradation due to high acidic nature of the leaf samples in *P. emblica* could be easily overcome by simple method of regulating the pH during the process of DNA isolation. The present protocol of DNA isolation in *P. emblica* is expected to be useful for ensuring the stability of pH in the buffer as rapid decrease of pH mostly occurred during grinding and incubation of samples at 65ºC.

P. emblica is one of the important agricultural commodities in India for domestic and international markets for wide range of food and pharmacological products. There are several commercial varieties of *P. emblica* (Indian Gooseberries) were produced and extensively cultivated in several parts of India. Nutritional properties of several varieties of *P. emblica* have been characterized and these varieties showed wide range of variation with regard to different agronomic features (Ojha and Pathak, 1993; 1992). There had been a demand for Indian varieties of *P. emblica* since promising varieties of *P. emblica* have not been developed in any other countries (Pathak *et al*., 2003) Thus, DNA fingerprinting of commercial varieties of *P. emblica* is very important in the context of plant variety protection for securing Intellectual Property Rights (IPRs). Our improved protocol of DNA isolation is expected to be useful for molecular characterization of several varieties of Indian gooseberries. In conclusion, our present study describes an easy and reliable protocol for isolation of DNA from leaf tissues of *P. emblica* for molecular characterization. The quantity and quality of the DNA is found adequate for molecular studies. The present protocol also yielded reasonable quality of chloroplast DNA and found suitable for amplification of chloroplast genes.

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