RESEARCH ARTICLE

A Standardized Protocol for Genomic DNA Isolation from the Species of *Plumbago* Linn.

Aswathy Ravindran[1], Jyotsna Gayan[2], Bandana Nabis Das[3*]

[1]P.G Student, Department of Botany, Hanidque Girls College, Guwahati Assam India

[2]Research Scholar, Institutional Biotech Hub, Handique Girls College, Guwahati Assam India

[3]Associate Professor, Department of Botany, Handique Girls College, Guwahati Assam India

[*]**Address for Correspondence:** Dr. Bandana Nabis Das, Associate Professor, Department of Botany, Hanidque Girls College, Guwahati Assam-781001, India

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ABSTRACT- A good amount of DNA can be extracted from the species of *Plumbago* Linn. by following the standardized and modified protocol. The DNA isolated was quantified using a spectrophotometer at the absorbance of λ_{260} nm and λ_{280} nm. Both the species given the best result with a DNA yield of 23.8µg/µl and 0.895 µg/µl in *P. zeylanica* and *P. indica* respectively and produced a clear band on the agarose gel. The estimation of purity of the obtained DNA was concluded by analyzing the ratio of λ_{260} nm/λ_{280} nm of the Genomic DNA, which was 1.94 and 2.0 for *P. zeylanica* L. and *P. indica* L. respectively. These results are of high quality as it was in the range of 1.8-2.0 by the present optimized protocol.

Key-words- Genomic DNA standardized protocol plumbagin

INTRODUCTION

Plumbago Linn. is a genus under family *Plumbaginaceae* consisting 10–20 species of flowering plants. They are widely distributed in the tropics [1]. In India the plant has a wide distribution ranging from Central India to North-east India, and various parts of Southern India. *Plumbago zeylanica* L. and *Plumbago indica* L. are the two medicinally important, morphologically different species of this genus selected for the present study. These two species has been reported from North-eastern regions of India. [2]

P. zeylanica L. is a multipurpose medicinal herb, commonly known as Ceylon leadwort or wild leadwort [3], however, its trade name in India is Chitrak. In north-east India it is widely distributed in the wilds of Assam, Meghalaya. Roots are widely used for its medicinal properties. Roots contain napthaquinone derivatives, plumbagin, chloroplumbagin, biplumbagin, elliptinone, chitranone [4]. Plumbagin shows strong antibacterial activity against *Escherichia coli, Salmonella typhi and Staphylococcus aureus,* and antifungal and inhibitory outcome in contradiction of *Klebsiella pneumoniae, Serratia marcescens* and moderate result in contradiction of *Bacillus subtilis,* and less low effect contrary to *Proteus vulgaris* and *Pseudomonas aeruginosa,* and aqueous extract shows the less antibacterial action as associated with organic extract.

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Plumbagin was shown anticancer, antimicrobial activity and antibiotic effects. [5]

Flowers are white in colour [4,7]. They are bisexual, regular, pentamerous, pedicellate and sweet-scented. The flowers are also characterized by having a tubular calyx (7–11 mm long and 5-ribbed) with glandular trichomes (hair) secreting a sticky mucilage. Flowers are insect pollinated. The mucilaginous glands aid in trapping insects and fruit dispersal by animals. The fruit of the plant is an oblong (7.5–8 mm long) five-furrowed capsule containing single seed. Roots are light- yellow when fresh and become reddish-brown on drying. The roots have a strong and characteristic odor [6]. Root extracts are effective against dysentery, diarrhea, diuretic, and peptic ulcers and intestinal parasite [7], malaria, rheumatism, intestinal parasites, anemia due to 'stagnant blood', internal and external trauma, toxic swelling and furunculous scabies can be treated with this plant [8]. The roots of the plant have abortifacient and vesicant effects. Paste prepared from the roots of the plant is applied to the skin to treat abscesses, other skin diseases including ulcers and scabies also [9]. Plant extracts have shown potent mosquito larvicidal activity against the larvae of *Aedes aegypti* while showing no toxicity to fish [10]. Hexane extracts of *P. zeylanica* have shown activity against canine distemper virus [11].

Plumbago indica L. is a medicinal herb and also planted in gardens as an ornamental plant. They are commonly known as Indian leadwort, scarlet leadwort, however, the trade name in India is Lal Chitrak. It is known for its medicinal properties in roots which contains Plumbagin (5-hydroxy-2-methylnaphthalene-1,4 -dione), sitosterol glycoside. Perennial herb or small shrub up to 2m tall. Flowers bisexual, regular, pentamerous. In *P. indica* L.

fruits have never been found. Especially the roots have many uses: it is acrid, vesicant, alternative, digestive, stimulant and a powerful abortifacient and oral contraceptive. High doses are dangerous and may cause death. An infusion of the roots is taken to treat dyspepsia, colic, cough and bronchitis. A liniment made from bruised root mixed with vegetable oil is used as a rubefacient to treat rheumatism and headache. The milky juice of the leaves is applied on the skin in the treatment of scabies, ring worm and haemorrhoids. *P. wissii* is a species in this genus, which is endemic to Namibia and tagged as 'Least Concern (LC)' by IUCN Red list [12].

Although the micro-propagation was the best way to preserve these species, the Genomic DNA isolation and its sequencing followed by comparing and finding out the various functions of its gene would reveal the most of the reasons behind the susceptibility of *P. indica* L. then *P. zeylanica* L. under environmental stress. Since the plant is ethno-medicinally important, a lot of human exploitation, particularly in Northeastern parts of India is occurring. Therefore the present study aims at providing a better survival of these species of *P.* Linn. by standardizing the protocol for the genomic DNA isolation in more quantity and in more purified form by doing a comparative analyzing between protocols for DNA isolation using CTAB by Doyle & Doyle [13] (modified) and Khanuja *et al.* [14] (modified).

MATERIALS AND METHODS

This study was conducted in the Institutional Biotech Hub and Department of Botany, Handique Girls' College, Guwahati, Assam, India in duration of six months from January – June 2016.

Plant material: Sapling of *P. zeylanica* L. were collected from different areas of Greater Guwahati, Assam, India and that of *P. indica* L. were collected from wild habitats of Amsoi hills, Nagaon, Assam, India and from Experimental Garden, Barapani; areas of Meghalaya and were raised under the Departmental botanical garden. Young and fresh leaves were harvested for DNA isolation.

Solutions

- 2% CTAB (100 ml)- extraction buffer
  2 g of CTAB were dissolved in 70 ml water, followed by the addition of 8.18 g of NaCl, 10 ml of Tris (pH 8.0; 1M), 4 ml of EDTA (pH 8.0; 0.5M). The volume was made up to 99 ml with water. Autoclaved, then added 1 g of PVP.
- Chloroform: Isoamyl (24:1) & Chloroform- 48 ml
  Isoamyl alcohol- 2 ml
- Isopropanol
- 70% Ethanol & 95% ethanol- 73.6 ml
  Sterile ddH_2O- 26.4 ml
- 0.1X TE buffer [100 ml; pH 8]
  100 µl of 1M Tris (pH 8) and 20 µl of 0.5 M EDTA (pH 8) was added to the water, followed by making up the volume to 100 ml and autoclaved.
- Ribonuclease A (10 mg/ml).

DNA isolation protocol

1. 1 gm of young and fresh leaves were weighed and allowed to freeze in liquid Nitrogen.
2. The frozen tissues are grinded into fine powder with the aid of a chilled sterile mortar and pestle in the presence of liquid nitrogen.
3. 200 mg of the powder was weighed and transferred in centrifuge tube using chilled spatula. Equal volume (w/v) of hot (65°C) 2% CTAB buffer was added to the powdered sample.
4. Mixed well by inversion and kept in water bath at 60°C for 1 hour with mixing well by inversion at every 15 minutes.
5. Equal volumes of Chloroform: Isoamyl (24:1) was added to the sample and mixed gently by inversion.
6. The mixture was centrifuged at 7280 g for 15 min. at 25°C–28°C.
7. The top aqueous layer was transferred to fresh tube and equal volume of chilled Isopropanol (-20°C) was added to the sample. On mixing it is gently shown a clear clouded clump of nucleic acids.
8. The mixture was centrifuged for 15 min at 7280 g.
9. A clear pellet was visible down the tube. The liquid was decanted and leaving the pellet attached to the wall of the tube.
10. The pellet was washed with 70% ethanol at 4°C in centrifuge at 7280 g for 15 min.
11. The decanted tube was air dried and the pellet was dissolved in 70 µl–100 µl of 0.1X TE buffer.
12. RNase treatment was given to the dissolved pellet by adding 8 µl RNase and kept in water bath at 55°C for 30 min–1 hour.

Estimation of DNA quantity and purity

The quantification and estimation of purity of the obtained genomic DNA per gram of the plant material was done using UV-VIS Spectrophotometer in the following steps-

1. 2 ml TE Buffer was taken in a cuvette and calibrated between λ_{260} nm and λ_{280} nm.
2. TE buffer was taken as blank.
3. 20µl of DNA sample was diluted into 1980 µl TE buffer. Therefore the dilution factor was 100.
4. Noted the OD λ_{260} nm and OD λ_{280} nm values of the spectrometer.
5. Calculated the OD λ_{260}/OD λ_{280} ratio to measure the purity of the obtained DNA sample.
6. The amount of DNA was quantified using the formula

$$\textbf{DNA concentration (µg/µl)} = \frac{\textbf{OD}_{260} \times 100(\text{DF}) \times 50}{1000}$$

RESULTS AND DISCUSSION

A comparative analysis between the present modified method of Khanuja *et al.* [14] and the standard method of Doyle & Doyle [13] draws clear and contrast difference in both the amount and purity of DNA isolated from the two

species of *Plumbago* Linn: *P. zeylanica* L. and *P. indica* L. The observations and results obtained after following the two methods are as follows-

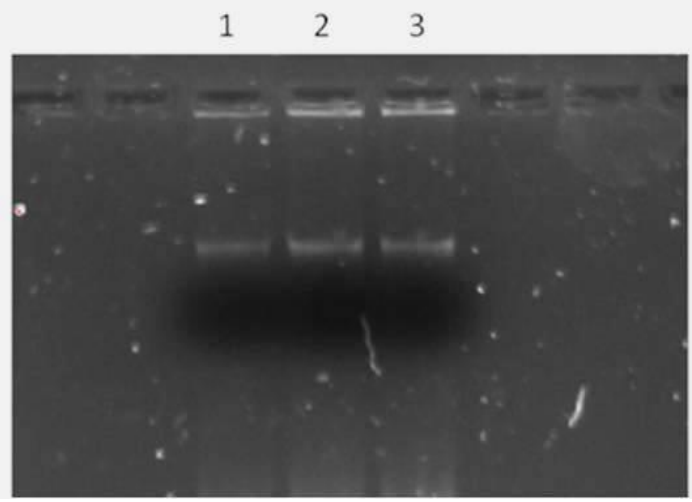

Fig. 1: Well No. 1&2 Loaded with sample extracted from *P. zeylanica*,
Well. No. 3 Loaded with sample extracted from *P. indica* By Modified method of Doyle & Doyle [13]

Method: I: Doyle & Doyle [13] (Modified)

The following observations were recorded under this method-

1. By following this method very less amount of DNA were able to extract. The width and dense of the band shows the least amount of Genomic DNA extracted through the modified method of Doyle & Doyle [13].
2. While performing the steps in the protocol pellets were barely visible.
3. Spectrophotometer analysis showed the DNA concentration to be 1.15µg/µl from *P. zeyalnica* L. and 0.62µg/µl from *P. indica* L. (Table 1).
4. The ratio of λ_{260}nm/λ_{280}nm of the Genomic DNA extracted by this method is 0.6, and 0.4 for *P. zeylanica* L. and *P. indica* L. respectively. (Table 1)

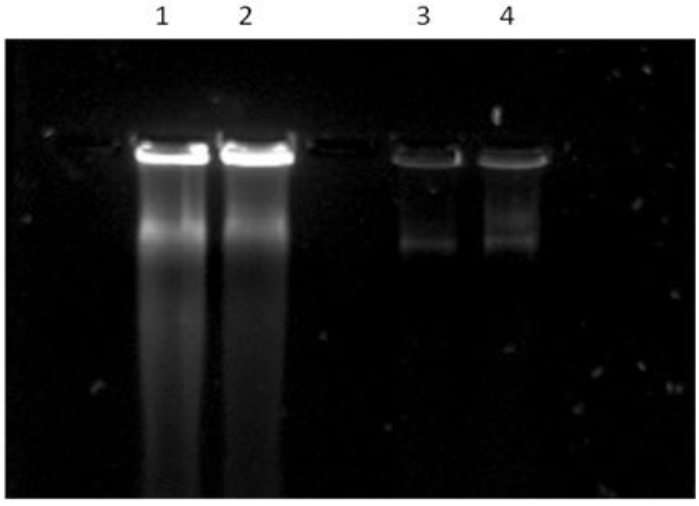

Fig. 2: Well No. 1 & 2 Loaded with sample extracted from *P. zeylanica*
Well No. 3 & 4 Loaded with sample extracted from *P. indica* by Modified method of Khanuja *et al.* [14]

Method: II: Khanuja *et al.* [14] (Modified)

The following observations were recorded under this method-

1. By following the modified method of Khanuja *et al.* [14], a noticeably larger amount of Genomic DNA bands was observed in the agarose gel electrophoresis analysis.
2. A thick coagulated mass was observed at the step when chilled Isopropanol were added in this method, which on centrifugation settled into a pellet.
3. Spectrophotometer analysis showed the DNA concentration to be 2.38 µg/µl from *P. zeyalnica* L. and 0.895 µg/µl from *P. indica* L. (Table 1).
4. The ratio of λ_{260} nm/λ_{280} nm of the Genomic DNA extracted by this method was 1.94 and 2.0 for *P. zeylanica* L. and *P. indica* L. respectively (Table 1).

The quality and quantity of the DNA was checked using a spectrophotometer. The quantification of DNA was done by recording its absorbance at λ_{260} nm to λ_{280} nm using a UV-VIS Spectrophotometer-

- The extracted DNA of the two species of *Plumbago* Linn: *P. zeylanica* L. and *P. indica* L. with the modified protocol of Khanuja *et al.* [14], was of high quality as its ratio of λ_{260}/ λ_{280} was 1.94 and 2.0, which was in the range of 1.8–2.0, in comparison to the yield of DNA by the modified protocol of Doyle & Doyle [13] where the ratio of $\lambda_{260}/\lambda_{280}$ was 0.6 and 0.4 in respectively.
- The absorption in the UV range λ_{260} nm was due to nucleic acids and the obtained yield was 2.38 µg/µl and 0.895 µg/µl form Khanuja *et al.* [14] (Modified) and from Doyle & Doyle [13] (Modified) only 1.15 µg/µl and 0.62 µg/µl in *P. zeylanica* L. and *P. indica* L. respectively (Table 1).
- In the present study selective precipitants viz, addition of 1g PVP in Extraction buffer, fixing the centrifugal force to 7820 g, fixing the incubation period to 1 hour with mixing well by inversion at every 15 minutes, etc were added/modified to remove the contaminants one by one and yield pure DNA, modification in centrifugation speed and time was also found effective in the yield of DNA.

Table 1: DNA obtained from different species of *Plumbago* Linn. using Doyle & Doyle [13] (modified) and Khanuja *et al.* [14] (modified)

	Modified method of Doyle & Doyle [13]				Modified method of Khanuja *et al.* [14]			
Species	**Optical density at λ_{260} nm**	**Optical Density at λ_{280} nm**	**Optical Density $\Lambda_{260}/\lambda_{280}$**	**DNA conc. µg/µl**	**Optical Density at λ_{260} nm µg/µl**	**Optical Density at λ_{280} nm**	**Optical Density $\Lambda_{260}/\lambda_{280}$**	**DNA conc. µg/µl**
***Plumbago zeylanica* L.**	0.23	0.345	0.6	1.15	0.476	0.245	1.94	2.38
***Plumbago indica* L.**	0.124	0.256	0.48	0.62	0.179	0.087	2.0	0.895

It was interesting to note that, except in some steps both species reacted to the reactants in the same manner.

- The first exception came to notice was their interaction with the CTAB extraction buffer (60°C). The powdered leaves of *P. zeylanica* L in liquid nitrogen interacted very well with the CTAB extraction buffer without changing its fresh green colour and its powdered particles nicely got spread with the buffer, on the other hand the powdered leaf samples of *P. indica* L. interacted with the CTAB extraction buffer in a more clumped manner, i.e. in contact with the CTAB buffer they got organized in a clumped form. With repetitive tapping and mixing by vigorous inversion only they got unclamped and allowed the extraction buffer to act on them. *P. indica* L. during the 1 hour incubation time with the CTAB extraction buffer changed its colour from green to lead (grayish black) color.
- The second exception was the time taken in the RNase action on the individual species. In *P. zeylanica* L. the RNase took 30 min. to degrade its RNA contaminant and on the other hand *P. indica* L. took 1 hour to totally degrade the amount of RNA present in the sample as a contaminant in 55°C as the suitable temperature.

The statistical analysis has shown the DNA concentration obtained by following the protocols of Doyle & Doyle [13] (modified) and Khanuja *et al.* [14] (modified). In the graph a clear, noticeable difference of the yield of the DNA from each protocol is observed. Therefore the method of Khanuja *et al.* [14] (modified) protocol is best suited to obtain genomic DNA from *Plumbago* Linn (Fig. 3)

The statistical analysis of the Extracted DNA purity by the two methods- Doyle & Doyle [13] (Modified) and Khanuja *et al.* [14] (modified) was observed in this Graphical representation of the ratio λ_{260} nm/λ_{280} nm. This ratio clearly shows the purity of the obtained DNA yield. From the graphical representation it was clear that Khanuja *et al.* [14] (modified) protocol was best suited to obtain a purified DNA yield from *Plumbago* Linn (Fig. 4).

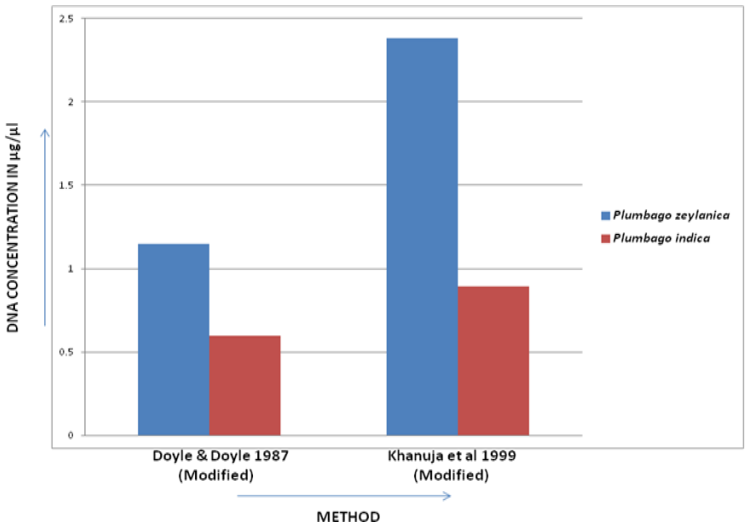

Fig. 3: Bar diagram showing DNA concentration

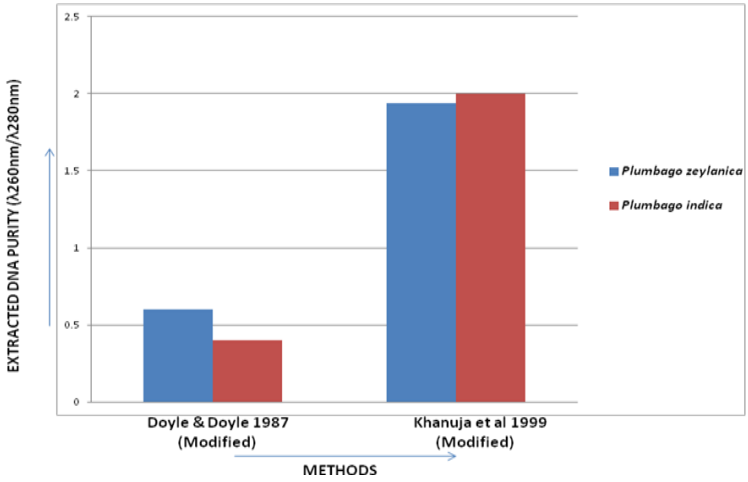

Fig. 4: Bar diagram showing extracted DNA purity

From the above two statistical representation it can be suggested that in *Plumbago* Linn, *P. zeylanica* L. genomic DNA was obtained in higher quantity than *P. indica* L. in both the protocols- Doyle & Doyle [13] (modified) and Khanuja *et al.* [14] (modified) with a p-value 0.0455. This may be due to the presence of more interfering secondary metabolites in *P. indica* L. than in *P. zeylanica* L. (Fig. 3, Fig. 4). Selective percipients like

extraction buffer, PVP etc were added to remove the contaminants one by one and yield pure DNA, modification in centrifugation speed and time also found effective in the yield of DNA. The extracted DNA was of high quality as it ranges from 1.94 to 2.0 in *P. zeylanica L.* and *P. indica* L. respectively.

Successful isolation of RNA free DNA was found to be 2.38 µg/µl and 0.895 µg/µl. after following the present optimized protocol of Khanuja *et al.* [14] with modifications.

CONCLUSIONS

A good amount of DNA can be extracted from *Plumbago* sp. by following the protocol of Khanuja *et al.* [14] (modified). Both the species gave the best result with a DNA yield of 2.38 µg/µl and 0.895 µg/µl and the ratio of λ_{260} nm/λ_{280} nm of the Genomic DNA was 1.94 and 2.0 for *P. zeylanica* L. and *P. indica* L. respectively. The estimation of purity of the obtained DNA was concluded by this result that demonstrated that genomic DNA ranging from 1.8-2.0 after following the present optimized protocol was of high quality. Fresh, tender and new leaves are mandatory as they contain secondary metabolites in less content and they are believed to be resistant to other parasites. Therefore, it can be concluded that this was the best suited protocol for genomic DNA isolation from *Plumbago* Linn.

Therefore further study and analysis of the genomic DNA of *Plumbago* Linn. will help to develop genetically engineered plants which will lead to more enhanced production of plumbagin and would have more resistance to environmental stress and secure survival demonstrated possible to this precious and ethno-medicinally important plant species.

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