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PCR-RFLP of *Calotropis gigantea (L)*- A Tool for Forensic Application

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Abstract

Plants besides having immense economic and medicinal value are also known to have poisonous products which are found in leaves, stem, bark, seeds, fruits, roots, flower, latex and gum exudates of such plants. These are used for cattle poisoning and sometimes as homicidal and suicidal agents. The major challenge for the forensic scientists lies in identification of the source of poisoning. In the medico-legal plant poisoning cases, identification of plants are based on exomorphological characters and on the basis of phytochemical and biochemical markers viz. secondary metabolites. However, the morphological characters changes with change in the environment, hence less reliable whereas secondary metabolites, may not be present in all the plant parts and thus cannot be used as authentic markers. Most effective approach to identify the source of plant poisoning is by using DNA markers, occurs naturally in all plants, does not change during life time for solving complicated criminal cases. The present study was undertaken to develop DNA markers using PCR-RFLP of *Calotropis gigantean,* a common roadside plant a potent poison. Internal Transcribed Spacer (ITS) region of the rRNA gene was PCR amplified and an amplicon of 770bp was obtained from four plant samples collected from different geographical regions. The amplified gene was subjected to restriction digestion with *HinfI, MspI, MboI, EcoRV, AluI, BamHI*, *EcoRI* enzymes. The generated DNA fragments in the size of 300,170,150,100 and 50 bp by *HinfI*; 380,190,150,50 bp by *MspI*; 720 & 50 bp in *MboI* and 450 & 320 bp in *EcoRV* can be applied for identification of *Calotropis gigantea* in forensic investigation in plant poisoning cases.

Keywords: *Calotropis* , Poisonous Plant, PCR-RFLP, Forensic

1. INTRODUCTION

Plants besides having immense economic and medicinal value are also known to have detrimental effect as poisonous products[1] which are found in leaves, stem, bark, seeds, fruits, roots, flower, latex and gum exudates of such plants [2]. These are used as criminal weapons for cattle poisoning and sometimes as homicidal and suicidal agents. The major challenge for the forensic scientists lies in identification of the source of poisoning. In the medico-legal cases involving seed poisons identification is based on the distinct diagnostic exomorphological characters and for others on the basis of phytochemical and biochemical markers viz. secondary metabolites. Wide ranges of chemical compounds consisting of alkaloids, flavonoids, tannins, saponins have been reported from *Calotropis* species[3]. But these flavonoids, saponins have also been extracted from other plants example leguminous plants[4]. Twelve well known common poisonous seeds have been studied on exomorphological basis, both by light microscopy and scanning electron microscopy[5], including *Calotropis gigantea, Datura innoxia*. It provides immense help in the detection of crimes involving poisoning cases. But due to environment of stomach, these morphological characters changes rapidly hence are of less significance. In such conditions biochemical markers can be taken use of, for example in hemp plants having poisonous seeds, can be differed from marijuana plants which have psychoactive drug tetrahydrocannabinol (THC)[2]. However these compounds are present in other plants also and thus cannot be used as markers. Similarly most of the phytochemical and biochemical markers are secondary metabolites which may not be present in all the plant parts and during whole life cycle. Hence might not be a full proof identification marker. There should be some other effective approach to identify the source of plant poisoning. Most effective approach to identify the source of plant poisoning in criminal cases is by using DNA markers, occurs naturally in all plants, specific to all species, does not change during life time. Different DNA markers used in plant genome analysis are RFLP, RAPD, AFLP, VNTRs, STRs[6,7,8,9,10]. AFLP markers can be used in discriminating illegal use of plants[2]. AFLP markers can also be used in forensic identification to link a piece of evidence with a particular location or suspect[11] . Other DNA markers like RAPD can be used to identify Strawberry varieties having forensic application[12]. Combination of plastidial and nuclear markers to identify toxic plants has been recommended[13]. The present study was undertaken to develop DNA markers using PCR-RFLP of Internal Transcribed Spacer(ITS) region of rRNA gene in *Calotropis gigantea* a common roadside plant having certain medicinal value but is a potent poison.

2. MATERIALS AND METHODS

Plant samples (leaves) were collected from five geographical region of northern India viz: Chandigarh (chd), Amritsar (amrt), Jagadhari (jg), Southern Delhi (SD) and Haryana (hry).

2.1 Isolation of plant DNA by CTAB method:

Plant DNA was extracted from leaves using modified CTAB method without liquid nitrogen[14]. The DNA of the isolates was electrophoresed using 0.8% agarose gel on Submarine Electrophoretic apparatus (Agagel Mini Biometra, Germany). The gel with 5mg/ml ethidium bromide was loaded with 3µl sample and 1µl Bromophenol blue (6X) as the loading dye and was exposed to a voltage of 55-60V for 45mins. The marker used was λ *Hind* III. The image was taken using the Gel documentation system Ultra Lum Gel imager (Alpha Innotech, DSS Alpha image, USA) (Figure-1 & 2). RNA was removed by adding Ribonuclease A (10µg/ml) and incubating at 37° C for 2hrs followed by chloroform : isoamyl alcohol (24:1) extraction and further precipitation of DNA. Highly pure DNA was obtained.DNA was quantified at 260nm using a double beam (UV-Visible) spectrophotometer (Systronics India Ltd).

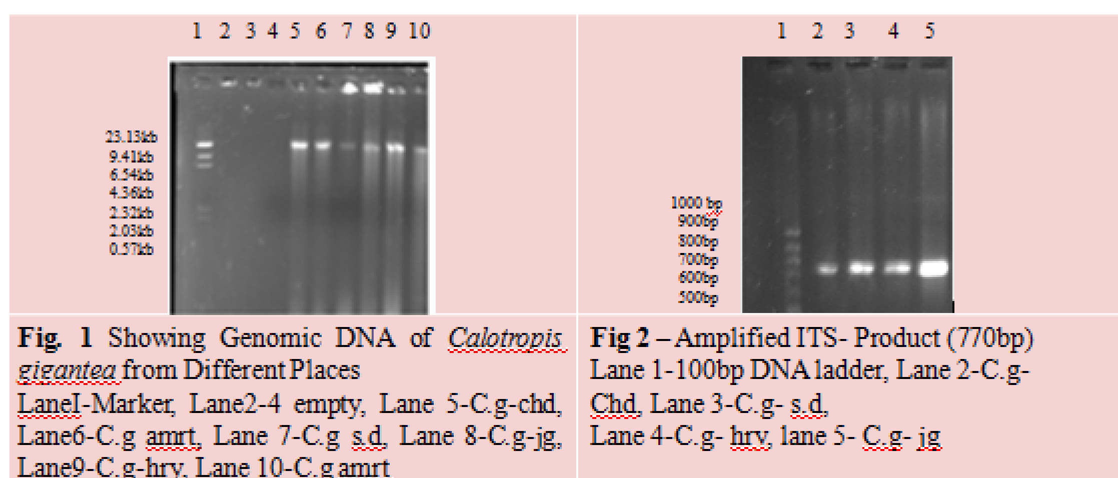

Fig. 1 Showing Genomic DNA of *Calotropis gigantea* from Different Places
LaneI-Marker, Lane2-4 empty, Lane 5-C.g-chd, Lane6-C.g amrt, Lane 7-C.g s.d, Lane 8-C.g-jg, Lane9-C.g-hry, Lane 10-C.g amrt

Fig 2 – Amplified ITS- Product (770bp)
Lane 1-100bp DNA ladder, Lane 2-C.g-Chd, Lane 3-C.g- s.d,
Lane 4-C.g- hry, lane 5- C.g- jg

2.2 PCR reaction mixture:

PCR amplification was carried out using Forward primer (ITS F) 20pM (5' TCC TCC GCT TAT TGA TAT GC 3') and Reverse primer (ITS 1F) 20pM (5' AAG TCG TAA CAA GGT TTC CGT AG 3'). Different PCR amplification cycles are -Initial denaturation of 95° C for 5min followed by, 30 cycles of 95° C for 30 sec , 52° C for 30sec , 72° C for 1min and then final extension period of 7 min at 72° C, after 30 cycle 04° C hold. PCR reaction mixture was set by using 47µl of PCR reaction mixture + 3µl of DNA sample making total volume equal to 50µl for each reaction. The quantity and concentration of various components used to prepare 50µl PCR reaction mixture are- Template DNA-3.0 µl ; 10x Taq buffer with 15mM magnesium chloride- 5.0 µl; $MgCl_2$(25mM) -1.0 µl(1.5mM); dNTP's (2mM) -5.0 µl ; Primer ITSF -1.0 µl; Primer ITS_1 F-1.0 µl; Taq Polymerase- 0.50 µl(1.25U/50 µl); Water-33.50 µl.

2.3 Restriction Digestion:

Restriction digestions were performed using ***MspI***, ***EcoRI***, ***HinfI***, ***MboI, Eco RV, AluI and BamHI*** to get restriction fragment length polymorphism for various gain and loss mutation at the respective restriction sites of ITS region. The amplified products were subjected to restriction by respective restriction enzymes. The amplified ITS products, enzyme buffers and autoclaved distilled water were thawed from -20 ° C to 4 ° C by placing on ice for 1 to 2 hrs. Mixtures were made for the enzymes as per the manufacturer instructions to act on the templates with the respective buffers, enzymes and distilled water separately for the enzymes working in different buffers. Each tube with the different enzymes consisted of the following components for a 20 µl mixture:

***MspI*:** Amplified ITS (template) 10 µl (1µg)+ Buffer (10X) 2 µl (1X) + Enzyme (10U/µl) 1 µl (0.5U/µl) + Autoclaved distilled water 7 µl; ***EcoRI*:** Amplified ITS (template) 10 µl (1µg)+ Buffer (10X) 2 µl (1X) + Enzyme (10U/µl) 0.75 µl (0.5U/µl) + Autoclaved distilled water 7.25 µl; ***HinfI*:** Amplified ITS (template) 10 µl (1µg)+ Buffer (10X) 2 µl (1X) + Enzyme (10U/µl) 0.3 µl (0.5U/µl) + Autoclaved distilled water 7.7 µl;

MboI: Amplified ITS (template) 10 µl (1µg)+ Buffer (10X) 2 µl (1X) + Enzyme (10U/µl) 0.5 µl (0.5U/µl) + Autoclaved distilled water 7.5 µl; ***EcoRV*:** Amplified ITS (template) 10 µl (1µg)+ Buffer (10X) 2 µl (1X) + Enzyme (10U/µl) 0.3µl (0.5U/µl) + Autoclaved distilled water 7.7 µl; ***AluI*:** Amplified ITS (template) 10 µl (1µg)+ Buffer (10X) 2 µl (1X) + Enzyme (10U/µl) 0.3 µl (0.5U/µl) + Autoclaved distilled water 7.7 µl; ***BamHI*:** Amplified ITS (template) 10 µl (1µg)+ Buffer (10X) 2 µl (1X) + Enzyme (10U/µl) 1 µl (0.5U/µl) + Autoclaved distilled water 7 µl. The mixtures were placed in the water bath (Tanco India) at 37 ° C for 16 – 20 hrs according to the time required by the enzymes for digestion. Care was taken that the mixtures were not incubated for long preventing over digestion. The mixtures were then placed in water at 65 ° C for 20mins to inactivate the enzymes. These were then electrophoresed in 2% agarose gel with 5µl sample and 1µl loading dye (as in DNA isolation procedure) and 100bp ladder as marker at 66V for 60-90 mins to check the RFLP patterns obtained by restriction digestion (Figure-3 and 4) of the enzymes on the ITS regions of the plant genome. Image of the restriction patterns was taken using the Gel documentation system Ultra Lum Gel imager. Restriction Fragment length (bp) polymorphism of ITS region in *Calotropis gigantea* after restriction digestion with different enzymes are presented in table-1.

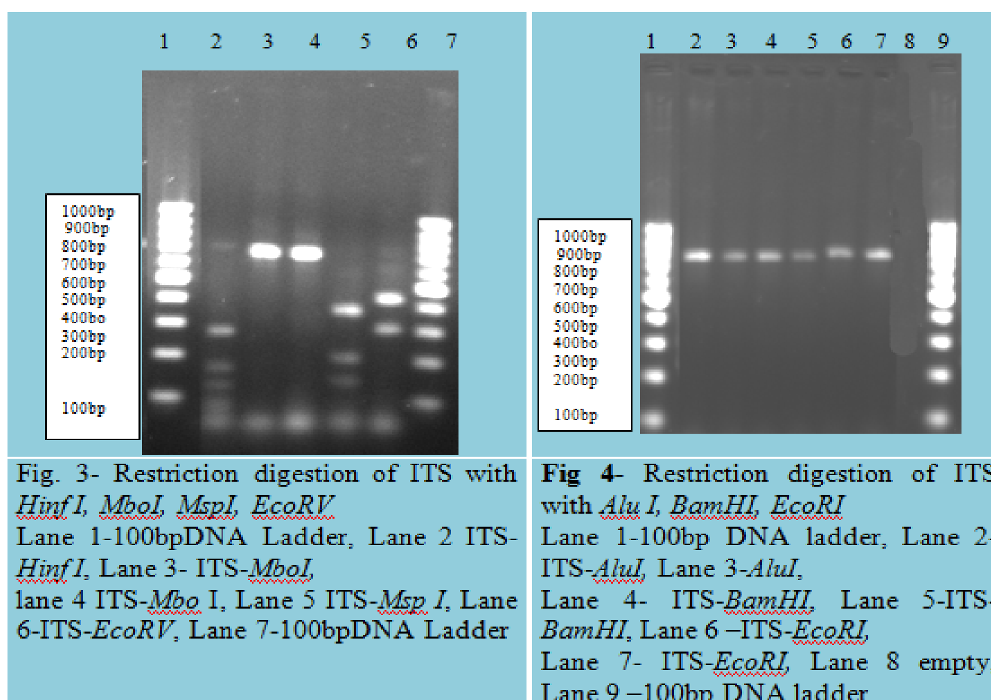

Fig. 3- Restriction digestion of ITS with *HinfI, MboI, MspI, EcoRV*
Lane 1-100bpDNA Ladder, Lane 2 ITS-*HinfI*, Lane 3- ITS-*MboI*, lane 4 ITS-*Mbo* I, Lane 5 ITS-*Msp I*, Lane 6-ITS-*EcoRV*, Lane 7-100bpDNA Ladder

Fig 4- Restriction digestion of ITS with *Alu I, BamHI, EcoRI*
Lane 1-100bp DNA ladder, Lane 2-ITS-*AluI*, Lane 3-*AluI*, Lane 4- ITS-*BamHI*, Lane 5-ITS-*BamHI*, Lane 6 –ITS-*EcoRI*, Lane 7- ITS-*EcoRI*, Lane 8 empty, Lane 9 –100bp DNA ladder

Table-1. Fragment length (bp) after restriction digestion with different enzymes

Plant	ITS	*HinfI*	*MboI*	*MspI*	*EcoRV*	*AluI*	*BamHI*	*EcoRI*
Calotropis Gigantean	770	300	720	380	450	770	770	770
		170	50	190	320			
		150		150				
		100		50				
		50						

3. DISCUSSION

All of the five samples of *Calotropis gigantea* produced the same 770 bp amplified ITS product showing that there is no difference in the size of their ITS region. *HinfI* and *MspI* generated 5 and 4 number of bands respectively. *MboI* and *EcoRV* generated two bands each. Whereas no digestion was observed with *AluI, BamHI* and *EcoRI* enzymes which could be attributed to two reasons that either there was no restriction sites of these enzymes in ITS region of rRNA gene or these sites may be methylated[15]. Similar results of no polymorphism was reported in ITS-RFLP of Cannabis species with restriction endonucleases *HinfI* and *EcoRV* [16]. In our study these enzymes did not show variation in *Calotropis gigantea* collected from different geographical region. As no RFLP was obtained between the samples, the specific banding pattern obtained with *HinfI* and *MspI* could be used as authentic marker for this plant from any region. The present work has established the RFLP based DNA

marker generated by amplifying the ITS region of *Calotropis gigantea* and followed by restriction digestion in combination of *HinfI*, *MspI, MboI* and *EcoRV* enzymes. The generated DNA fragments in the size of 300,170,150,100 and 50 bp by *HinfI*; 380,190,150,50 bp by *MspI*; 720 & 50 bp in *MboI* and 450 & 320 bp in *EcoRV* can be applied for identification of *Calotropis gigantea* in forensic investigation. Similar result was reported earlier in a case report[17]. In our earlier studies we have demonstrated the variations in the ITS region among species can be used for conclusive identification[9,10]. Apart from RFLP other DNA marker based studies like AFLP, RAPD, microsatellite fingerprinting as reported earlier could also be applied to get unique pattern. Absence of polymorphism using any of these enzymes in all the four samples of *Calotropis gigantea* collected from different places established that the DNA marker developed by the present work is not geographical region specific and can be used widely.

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