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An improved protocol for genomic DNA extraction and purification from leaves of *Terminalia Arjuna* (Roxb.) Wight and Arnis

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Abstract

Several available protocols fail to produce good quality genomic DNA from leaves of Terminalia arjuna that contain the exceptionally high amount of interfering secondary metabolites and polysaccharides. To address this issue, a modified DNA isolation protocol was developed for obtaining quality genomic DNA. Briefly, leaf tissue was macerated in lysis buffer supplemented with PVP, sorbitol and sodium chloride. The macerated material was washed in sorbitol buffer for easy extraction and getting rid of mucilaginous substances present in the sample. Subsequently, the inclusion of high concentrations of B-mercaptoethanol, NaCl, and Triton-x in Cetyltrimethylammonium bromide (CTAB) removed polyphenols and polysaccharides. Phenol: Chloroform: Isoamyl alcohol washings took after by precipitation with NaCl proficiently expelled high protein and polysaccharide impurity. The purity of the isolated genomic DNA was checked spectrophotometrically and confirmed by the running of isolated DNA on 0.8% agarose gel electrophoresis. The yield of pure genomic DNA ranged from 120 to 570ng/µl and purity index (A260/A280) ratio from 1.65 to 2.0. The isolated genomic DNA optimized the RAPD analysis and showed clear amplification in all the germplasm tested.

Keywords: *Terminalia arjuna*, DNA isolation, medicinal plants, Lysis buffer, RAPD.

Introduction

In Ayurveda, many medicinal trees are described. Among these trees, Arjun (*Terminalia arjuna* (Roxb.) Wight and Arnis known for thousands of years due to its medicinal properties. Its various parts are used in curing different ailments. However, the stem bark with the high medicinal value finds economic significance in pharmaceutical industries. The bark of Arjun is broadly used as astringent, cooling, antioxidant, cardiotonic, aphrodisiac, in fractures, diabetes, ulcers, tumor, spermatorrhae, leucorrhoea, cough, excessive perspiration, asthmatic and skin related problems. The pharmacological and clinical investigations of its unrefined medication constituents and different concentrates have exposed its implication in the treatment of anti-inflammatory, anti-rheumatic, cardiovascular disorder, hypercholesteremic, hypolipidemic, and antifertility activities[1]. Moreover, a hypolipidemic drug has been derived from Arjunbark containing triterpenoid along with other constituents and is being promoted marketed to be utilized as the part of the allopathic, Ayurvedic and Unani therapeutics medicinal framework systems to conflict human ailments[2].

Terminalia arjuna is found all over the country in deciduous forests such as those of Andhra Pradesh, Assam, Bihar, Gujarat, Jammu and Kashmir, Maharashtra, Karnataka, Tamil Nadu, Utter Pradesh and West Bengal[3-5].

The tree has a wide canopy which faces extinction due to over-harvesting of bark by traditional bark collectors. As a result, there is a drastic decline in the population of Arjuntrees in its natural habitat. Hence, there is an urgent need for conservation of wild germplasm either in situ or ex-situ.

For the purpose of successful breeding programs, proper identification of the plant is of prime importance, for which an accurate, reliable and more authentic system of classification is required. Conventionally, identification and classification of plant groups are solely based on similarity and dissimilarities in the morphological feature, more importantly, the floral character which was considered to be consistent. As has already been established, morphological characters are considered to be the expression of both gene(s) and environment and their interaction and often get greatly influenced by climatic and edaphic factors. Plant species belonging to the same or related genera can show colossalin consistency for performing complex life functions. Hence, the biochemical composition in plant tissues of same species growing in diverse locations is expected to vary considerably. Molecular techniques are especially helpful exclusively to distinguish the genotypes for the verification yet in addition in surveying and exploiting the inherited variability[6]. Michiels et al.[7] emphasized that the isolation of high molecular weight genomic DNA is important for starting any molecular biology work. DNA isolation from *T. arjuna* is famously troublesome due to the fact that it comprises high amounts of polysaccharides, polyphenols and other secondary metabolites like oleo-gum-resin which form insoluble complexes with nucleic acids in the course of extraction[8], posing difficulty during the time of DNA extraction. The polysaccharides are

visually marked by their gelatinous glue-like consistency and create the problems during the pipetting of DNA and also difficult to amplify by PCR[9]. Since plants contain a high measure of the extensive variety of various substances, it is very difficult that only one nucleic acid isolation protocol seemly suitable for all plants can ever exist[10]. A few DNA isolation protocols were striven[9,11-13] for obtaining genomic DNA from *T. arjuna*. All these protocols resulted in brown or yellow DNA precipitate with the low quality of DNA that could not be unfailingly amplified by PCR. Therefore, this circumstance requires the improvement of a reliable protocol for attaining higher quantity and highly purified DNA in *T. arjuna* that could be used for molecular biology work.

Materials and methods

Plant material: Young fully leaves from the morphologically superior and pathogen free trees of *T. arjuna* were collected from the Central Tasar Research and Training Centre of Ranchi for developing a reliable genomic DNA extraction protocol.

Reagents and solutions: HEPES- 1M, PVP-20% (w/v) was prepared, TRIS-Cl-1M (pH 8.0), EDTA- 0.5M (pH 8.0), NaCl-5M, SDS-20%, Ammonium Acetate-7.5M, Sucrose 25%, CTAB, Sorbitol, Triton, Phenol: Chloroform: Isoamyl alcohol (24:24:1), Ethanol, β-Mercaptoethanol, Triton x-100.

Lysis Buffer: Stock solution of 20% (v/v) was prepared by of 20% (w/v) PVP, 2% Sorbitol, 5 M NaCl.

Extraction buffer: 4% Hexadecyltrimethyl-ammonium bromide (CTAB) (w/v), 120 mMTris-Cl (pH 8.0), 50 mM EDTA (pH 8.0), 1 M NaCl, 25% Sucrose, 2% Triton autoclave and add 20% Polyvinylpyrrolidone (v/v), and 4% β-Mercaptoethanol instantly before use; care was taken at the time of addition.

Resuspension buffer: 20mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0).

TE Buffer: 10mM Tris-Cl (pH 8.0), 1mM (pH 8.0) and 3M NaCl.

Preparation of extraction buffers: The extraction buffer used for the homogenization contained 120 mM Tris-Cl (pH, 8.0), 50 mM EDTA (pH, 8.0), 1M NaCl, 20% PVP and 2% Triton. The extraction buffer was autoclaved and 4% β-mercaptoethanol and 4% CTAB were included instantly before use. At the time of reagents preparation, care was taken to avoid the precipitation.

Protocol for genomic DNA Isolation: i. First wash the leaves with distilled water properly and weigh 150mg of the fresh plant leaf tissue and grind with the help of 1ml lysis buffer into a homogenous paste using a mortar and pestle, this homogenous paste was transferred in a 2ml Eppendorftube and add 1ml lysis buffer. The contents were kept emulsified for 2hours on a rocker set at low speed (50 revolutions per minutes) at room temperature to remove secondary metabolites. ii. The contents were centrifuged at 12000 rpm (Rotor- 220.87 V11, Eppendorf 5430R Tabletop centrifuge) at 20°C for 10 minutes and the supernatant was discarded. iii. Wash the precipitant with wash buffer at 1200rpm for 3 minutes, the supernatant was discarded and add 1ml wash buffer and dissolve the precipitant. Same steps were repeated 4 to 5 times, to eradicate sticky residue from the precipitant. iv. One ml of extraction buffer was added and centrifuged at 12000 rpm for 5minutes. Eliminate the supernatant and add 450µl of resuspension buffer to the precipitant. 80µl 0f 20% SDS was added in this precipitant. The contents were smoothly mixed and incubated at 70°C for 30 minutes. v. The sample was placed at room temperature and after that when the sample becomes cool at room temperature 300µl of 7.5M Ammonium acetate was added and put the sample for 30 minutes on ice. vi. Spin the sample at 1200 rpm for 15 minutes. Carefully transfer the upper clear aqueous layer to a new 1.5ml Eppendorf tube. vii. This aqueous phase obtained from the previous step, an equal volume of ice-cold isopropanol was added. The contents were mixed softly by reversal to allow the DNA to precipitate. viii. The DNA strands obtained were then spooled out into a microfuge tube. To this 70% Ethyl- alcohol was added. This was allowed to stand for 30 minutes at 4^0C. The microfuge was centrifuged at 12000 rpm for 15 minutes and the supernatant was decanted carefully. The 70% ethyl alcohol wash was repeated once more. ix. The pellet was air dried to remove the traces of alcohol. The DNA was then dissolved in 100µl of TE buffer which contains 3M NaCl. Those samples that did not dissolve readily were kept at 60°C for 30 minutes to 1 hour.

Purification of DNA: i. DNase free RNase with a concentration of 10 µg/ml was added and the solution, an incubation was given to the solution at 37°C for one hour. ii. For the removal of RNase, the equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) were added. The two phases were mixed gently for 15 seconds and then centrifuged at 12000 rpm for 10 minutes at room temperature. iii. The upper aqueous phase was collected in a fresh microfuge tube. The equal volume of Phenol: Chloroform: Isoamyl alcohol (24:24:1) was added again. The two phases were mixed gently for 15 seconds and then centrifuged at 12000 rpm for 10 minutes at 4^0C. iv. In another microfuge tube, the upper aqueous phase was collected and mixed gently and the equal volume of ice-cold ethanol was added. The content was mixed by inversion and was left for 30 minutes to 1 hour at -20°C for DNA precipitation. v. The solution was centrifuged at 12000 rpm for 15 minutes at 4°C. A 70% ethyl alcohol wash as described in step 11 was performed. vi. The pellet was then air dried and dissolved in 50 µl of TE buffer.

Estimation of the DNA concentration: The concentration of the DNA purified by this method was determined by the comparison with the DNA ladderon 0.8% agarose gel. The concentration of the DNA was also determined by Nanodrop spectrophotometer absorbance at 260nm (for double stranded)

of genomic DNA sample (1OD $_{(A260)}$ = 50µg of double-stranded DNA/ml). The purity of the DNA sample was determined by A_{260}:A_{280} ratio (1.6-1.8 for pure DNA).

Optimization of RAPD reaction: DNA extracted by this method from the leaf samples of arjun was used for the optimization of RAPD reaction. RAPD decamer primerOPA20 with the sequence '5 "GTTGCGATCC" 3'was used to amplify these extracted DNA samples and for the standardization of the PCR conditions.

PCR amplification was done in 20µl reaction volumes containing 2.5µl of 1X assay buffer, (with 25mM MgCl2), 5 mM dNTPs (Himedia Laboratories, Pennsylvania US) 20pM of random decamer primer (GeNoRime Biotech Service Pvt Ltd, Chennai) 1.0 unit of Taq DNA polymerase and 50 ng of template DNA. Thermal Cycler (Eppendorf AG, Hamburg, Germany) was used for the amplification of DNA with the programmed for 40 cycles as follows: No of cycles – 40, Initial Denaturation - 94/5 min, Denaturation - 94/1 min, Annealing - 38/1min, Extension - 72/3 min, Final extension - 72/7 min, Soaking Temperature (ºC) – 4, 1st cycle 5 min (94ºC).

After completion of the PCR, 5 µl of 1X loading dye (MBI Ferment Inc., Maryland, USA) was added to the amplified products. 1.2% agarose was dissolved in 100ml of 1X TAE bufferand irradiated by microwave until agarose melt completely and gel appeared transparent. 5 ulethidium bromides (10mg/ml) was added in the gel and cast the gel and left for the solidification. Set up the unit and sample was loaded in the wells and run the electrophoretic unit buffer (Himedia Laboratories, Pennsylvania, US) with 1X TAE. Electrophoresed gels captured and predicted by a gel documentation system (Syngene, Cambridge, UK).Standard DNA ladder (Himedia Laboratories, Pennsylvania, US) was used for the estimation and comparison of the sizes of the amplified products.

Results and discussion

DNA extraction was improved by major modifications of the CTAB method[12]. The effect of Sorbitol, Triton-X, and PVP individually or in combination in CTAB buffer was considered by extracting DNA from *T. arjuna* samples. Concentrations of more or less than 500mM NaCl and 2% PVP incorporating in extraction buffer did not give the promising result for DNA precipitation (data not shown). Syamkumar *et al.*[14] evidenced that high concentration of CTAB and NaCl in extraction buffer gives the high yield of DNA in some plants. Fang *et al.* (1992)[15] reported that polysaccharides contaminations are adhesive as they can restrain the action of numerous commonly used molecular biology enzymes, such as polymerases for which increased concentrations of NaCl more than 1.5 M can be used for elimination of polysaccharides. A few strategies on the exclusion of polysaccharides from DNA have been broadly examined[16,17]. Katterman and Shattuck[18]; Peterson *et al.*[19] stated that the existence of polyphenols which are strong reacting agents bind covalently with the isolated DNA and can reduce the yield and purity and make the DNA unusable for most of the molecular biological works.

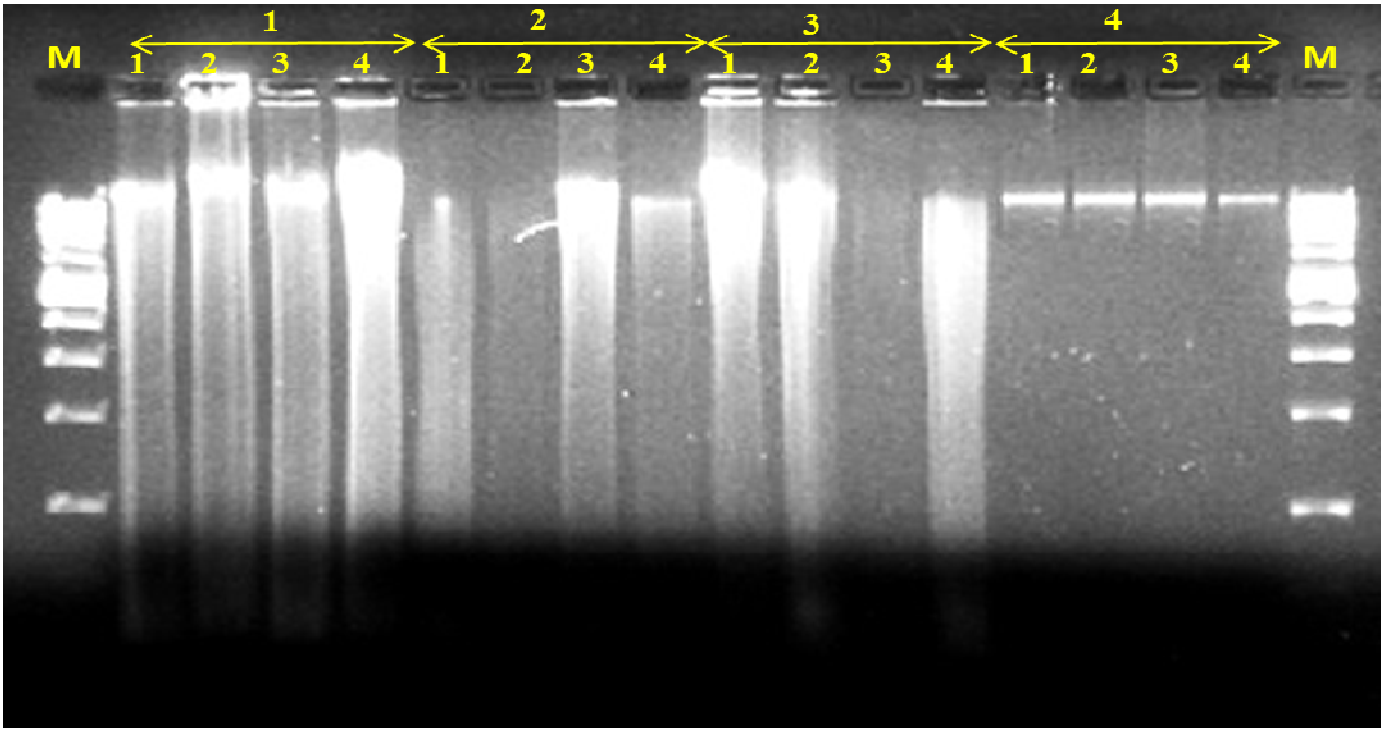

Figure-1: Genomic DNA extracted by the currently developed method of *T. arjuna.*

Table-1:

	Doyle & Doyle, 1990	Dellaporte et al.1983	Deshmukh et al.2007	Our Method
Crushing	CTAB buffer	Liq N_2	Liq N_2	Lysis buffer 20% (v/v) of 20% (w/v) PVP, 2% Sorbitol, 5 M NaCl
Wash buffer	–	–	100mM HEPES, 0.1 % PVP, 4 % BME (v/v)	–
Extraction buffer	–	50mM EDTA (pH 8.0), 100mM Tris-Cl (pH 8.0), 500mM Nacl, 0.7% BME	50 mM EDTA(pH 8.0), 50 mM Tris-Cl (pH 8.0), 500 mM Nacl, 15% Sucrose (w/v)	4%(CTAB) (w/v), 120 mM Tris-Cl, 50 mM EDTA, 1 M NaCl, 25% Sucrose, 2% Triton x-100, 20% PVP (v/v), 4% β-BME
Resuspension Buffer	–	10mM EDTA(pH 8.0), 50mM Tris-Cl(pH 8.0)	10mM EDTA (pH 8.0), 20mM Tris-Cl(pH 8.0)	20mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)
TE Buffer	DNase free water	0.5 M EDTA, 1 M Tris-Cl	1mM EDTA(pH 8.0),10mM Tris-Cl(pH 8.0)	1mM EDTA(pH 8.0), 10mM Tris-Cl(pH 8.0)
SDS	_	20%	10%	
NaOAC/NH_4OAC	7.5M (NH_4OAC)	3M (NaOAC)	7.5 M (NH_4OAC)	7.5 M (NH_4OAC)
Chf:IsoamyAlc.	24:1	_	24:1	24:24:1(Phenol:ChF:IsoamyAlc)
Isoprapanol	Absolute	Absolute	Absolute	Absolute
Ethanol	70%	80%	70%	70 %
DNA Purity (260/280)	–	1.04-1.35	1.17-1.55	1.6 - 1.8
DNA yield	_	271.3-1389.5	98.7-768.2	120.2- 570.0

Some workers found in their studies that DNA[20] contaminated with polyphenol looked resistant to restriction enzymes and it has also been reported in other plant species which have high amount of phenolic content and interrelate conclusively with proteins and nucleic acids[11]. This process is mainly because polyphenols oxidized into quinines and quinones by polyphenol oxidase took after by covalent coupling or by oxidation of the proteins by the quinines. During homogenization, polyphenols are discharged from vacuoles and afterward they respond quickly with cytoplasmic enzymes. The use of high molecular weight PVP (40,000) along with CTAB to extraction buffer remove phenolic compounds from plant DNA extracts, it forms H-bond with phenolic compounds, subsequently, CTAB is a powerful cationic surfactant and it disrupts membrane and releases DNA. The combination of these chemicals i.e. CTAB and PVP is used to minimize the effect of this secondary metabolite[14,20,21] and β-mercaptoethanol also reduces polyphenol oxidation[22].

In the same way, the addition of ethanol in the last step improves the DNA yield and purity because of ethanol change the ionic potential of DNA and remove water molecule, which helps in precipitation of DNA. Since DNA is insoluble in alcohol because of the polarity, during centrifugation it will aggregate together and form a pellet. This step is an important step because it removes alcohol soluble salt. It was observed that incorporation of 20% PVP, 2% Sorbitol to CTAB buffer provide good quality and quantity DNA detected by RAPD markers (Figure-2). Using this method, colorless DNA from these accessions of *T. arjuna* was achieved. So, we found in this study that previous methods was not suitable for the isolation of DNA from this species, the DNA isolated by these methods were impure and unusable for the further molecular work.

High quantity and good quality DNA was isolated successfully from *T. arjuna*using the protocol described above from 150 mg of fresh tissue. The isolated DNA had normal spectra in which the A260/A280 ratios were 1.65-2.0. From the Agarose gel electrophoresiswe depicted that DNA bands are clear and sharp and there is no evidence of any contamination of RNA and protein.This prove that DNA was of high molecular weight; the yield range was 120-570 ng/µl. The DNA isolated from accessions of *T.arjuna*using this method was suitable for RAPD experiments using OPA 20 (Figure-2).

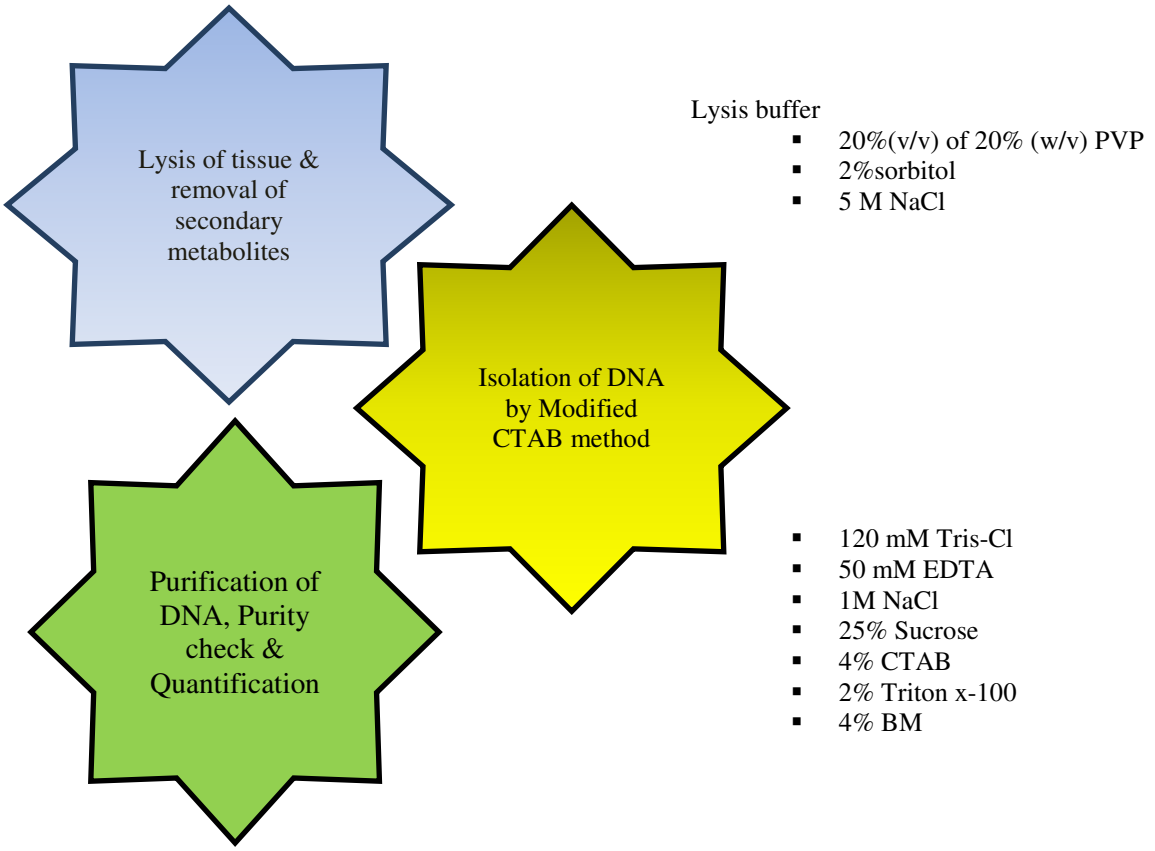

Figure-2: A flow diagram representing the whole protocol for isolation and purification of PCR usable genomic DNA.

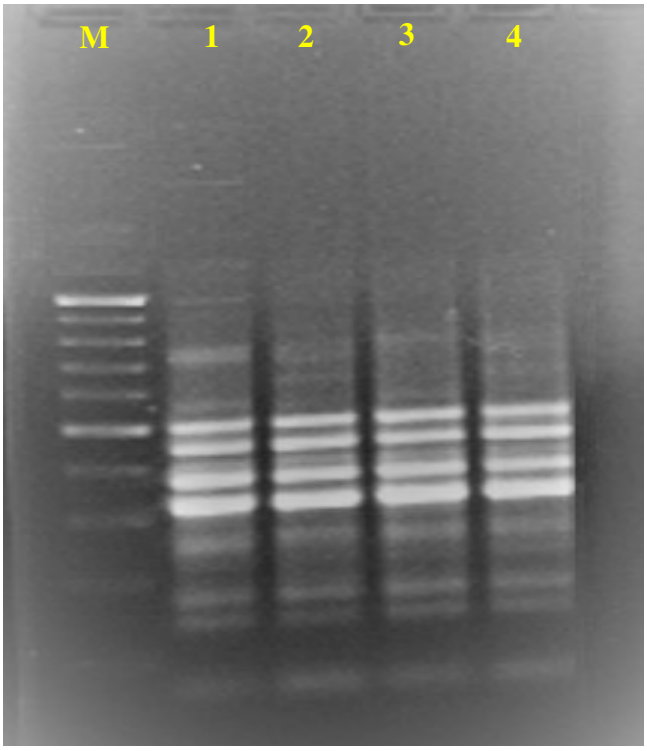

Figure-3: RAPD banding patterns of 4accessions of *T. arjuna*amplified with primer OPA 20 (5'GTTGCGATCC3') M- 1kb DNA ladder, Lane 1-4: leaf DNA isolated from accessions of *T. arjuna*.

Conclusion

Terminalia arjuna is medicinally a very important plant especially as a source of bioactive chemicals, used in the treatment of several diseases in different systems of medication. The plant faces a risk of endangerment due to over-harvesting of bark and other natural and man-made reasons which have been documented by various researchers. There is a drastic decline in the population of Arjun trees in its natural habitat. To replenish the declining population, systematic breeding programs with the input of molecular techniques are especially helpful exclusively to distinguish the genotypes having maximum quantity of active bio-chemicals and exploiting the inherited variability are the need of the hour. The isolation of high molecular weight genomic DNA is important for starting any molecular biology work. The existing protocols fail to produce good quality genomic DNA from leaves of *Terminalia arjuna* due to the presence of the exceptionally high amount of interfering secondary metabolites and polysaccharides. To address this issue, a modified DNA isolation protocol was developed for obtaining quality genomic DNA. The isolated genomic DNA optimized the RAPD analysis and showed clear amplification in all the germplasm tested. The present investigation on the advancement of the protocol for isolation of high quality and purity DNA and optimization of RAPD conditions is good for *Terminalia arjuna* without using liquid nitrogen to isolate DNA. This study will provide a new beginning for molecular characterization and genetic improvement works in this promising restorative medicinal plant. Such studies will be helpful to select and cultivate superior quality genotype, take up transgenic studies and conduct breeding experiments for the propagation of improved germplasm.

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