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TECHNICAL NOTE

A simple method for isolation of genomic DNA from fresh and dry leaves of *Terminalia arjuna* (Roxb.) Wight and Argot

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Keywords: Ayurveda, DNA extraction, medicinal plant, *Terminalia* species.

Abbreviations: PCR: polymerase chain reaction
RAPD: random amplification of polymorphic DNA

Current protocols for isolation of genomic DNA from *Terminalia Arjuna* have their own limitations due to the presence of high content of gummy polysaccharides and polyphenols. DNA isolated by these protocols is contaminated with a yellowish, sticky and viscous matrix. In our modified DNA isolation method polysaccharides and polyphenols are removed prior to the precipitation of the DNA. Then the genomic DNA was precipitated using isopropanol. This protocol yielded a high molecular weight DNA isolated from fresh as well as dry leaves of *T. Arjuna*, which was free from contamination and colour. Isolated DNA can be used for restriction digestion and PCR amplification. The main objective of the present protocol is to provide a simple method of isolation of DNA, using in house prepared reagents.

Terminalia arjuna (Roxb.) Wight and Arnot (family Combretaceae) is a large tree distributed throughout India and is known for its very high medicinal value in ancient Ayurveda and Unani medicine therapies (Pasquini et al. 2002). Herbal drug therapies are getting importance because of their easy availability and less side effects as compared to other medicinal therapies. In ayurvedic literature *T. arjuna* is known for its various medicinal properties like tonic, anthelmintic, styptic and alexiteric (Scassellati-Sforzolini et al. 1999). The bark of *T. arjuna* is known for treating heart diseases, coronary artery diseases

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and hypercholesterolemia (Miller, 1998). The plant has considerable importance as timber and its tannin containing nuts (Ohri, 1996).

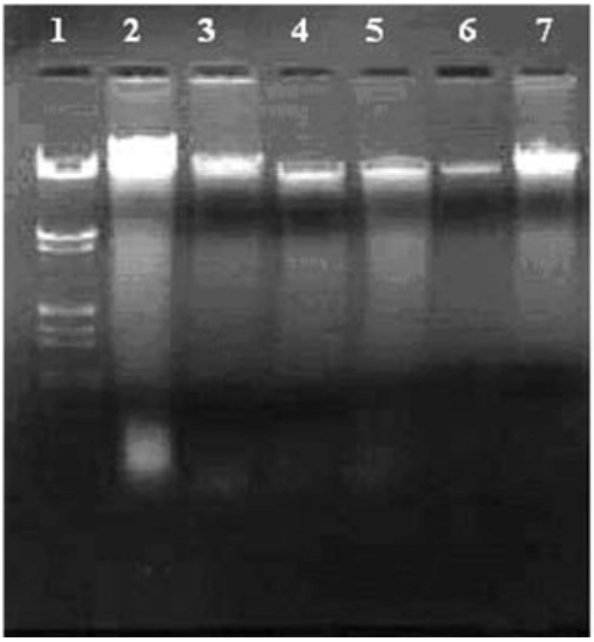

Figure 1. DNA isolated from fresh and dry leaves on 0.7% agarose gel from left to right. Lane 1: λ DNA Digested with Eco R I + Hind III; Lane 2: *Terminalia arjuna* fresh leaves; lane 3: *Terminalia arjuna* dry leaves; lane 4: *Terminalia tomentosa* fresh leaves; lane 5: Terminalia tomentosa dry leaves; lane 6: *Terminalia bellerica* fresh leaves; lane 7: *Terminalia chebula* fresh leaves.

Polyphenolic contents were reported from *T. arjuna* by Bajpai et al. (2005). A little is known about the molecular biology of *T. arjuna* (Bharani et al. 2002), due to the presence of polyphenolic and polysaccharide compounds, which acts as inhibitors during isolation of DNA. During the isolation of DNA from perennial plant tissue like leaves of *T. arjuna*, these inhibitory substances get precipitated along with the DNA, thus deteriorating the quality and yield of the DNA. To solve this problem, we tried several protocols, which were reported previously along with various modifications from both fresh and dry leaves, but none yielded DNA free from polysaccharides and polyphenols. These situations required the development of a new protocol for the isolation of genomic DNA of high purity from *T. arjuna*.

We describe a simple DNA isolation protocol that yields high quality genomic DNA from fresh as well as dry leaves of *T. arjuna*. The isolated DNA has proved amenable to polymerase chain reaction (PCR) amplification and restriction digestion. The proposed method makes use of in-house prepared and readily available reagents and thus provides an alternative to the use of commercial DNA isolation kits. This protocol is applicable to other similar species of family Combretaceae *e.g. Terminalia bellerica, Terminalia chebula* and *Terminalia tomentosa*.

MATERIALS AND METHODS

Collection of plant material

Juvenile leaves of *T. arjuna*, *T. bellerica*, *T. chebula* and *T. tomentosa* were collected from different locations of Melghat Tiger Reserve, Maharashtra, India (Latitudes 21º 15'N and 21º 45'N, Longitudes 76º 57'E and 77º 33'E and altitude 312 M to 1178 M above mean sea level), and brought to the laboratory in ice bags. The DNA was extracted from half the quantity of collected fresh leaves on the same day and the remaining half was kept at 50ºC for overnight.

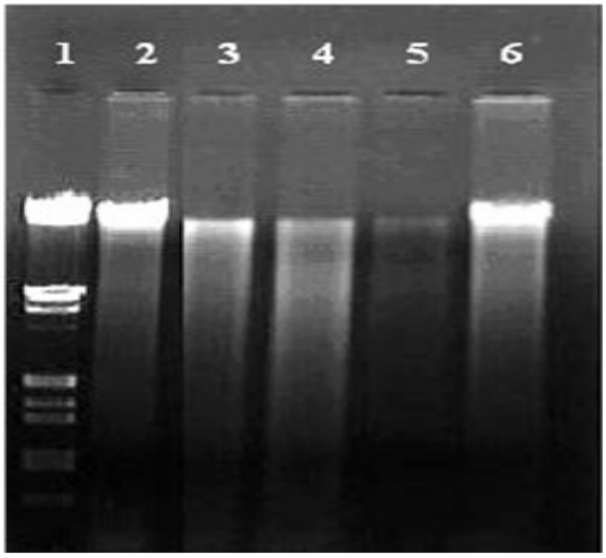

Figure 2. DNA sample digested with *Eco* R I enzyme from left to right. Lane 1, λ DNA digested with *Eco* R I and *Hind* III; lane 2: *Terminalia arjuna* fresh leaves; lane 3: *Terminalia tomentosa* fresh leaves; lane 4: *Terminalia tomentosa* dry leaves; lane 5: *Terminalia bellerica* fresh leaves; lane 6: *Terminalia arjuna* dry leaves.

Reagents and chemicals required

- HEPES (1 M); polyvinylpyrrolidone; β-mercaptoethanol; 25% sucrose; Tris-Cl pH 8.0 (1 M); EDTA pH 8.0 (0.5 M); NaCl (5 M); SDS (10%); ammonium acetate (7.5 M); isopropanol; ethanol; chloroform; isoamyl alcohol (24:1) (v/v).

- Wash buffer: 100 mM HEPES, 0.1% (w/v) polyvinylpyrrolidone, 4% β-mercaptoethanol (v/v) (added immediately before use).

- Extraction buffer: sucrose 15% (w/v), 50 mM Tris-Cl (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl.
- Resuspension buffer: 20 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0).

- TE Buffer: 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA (pH 8.0).

DNA isolation protocol

- Grind 300 mg of fresh leaves (for dry leaves 50 mg) in liquid nitrogen.
- Transfer the material to 1.5 ml microfuge tube and add about 1000 µl of wash buffer. Vortex the sample for 5 min to remove polyphenols.
- Spin at 12,000 g for 3 min.
- Remove the supernatant and repeat the washing step 4 to 5 times to take out sticky residues from the precipitant.
- Add 1000 µl of extraction buffer to the precipitant and centrifuge at 8,400 g for 5 min.
- Remove the supernatant and add 450 µl of resuspension buffer to the precipitant along with 80 µl of 10% SDS and incubate at 70ºC for 15 min.
- Allow the sample to cool to room temperature, then add 300 µl of 7.5 M Ammonium acetate and place the sample on ice for 30 min.
- Spin at 12,000 g for 15 min.
- Carefully transfer the upper clear aqueous layer to another 1.5 ml microfuge tube.
- Add equal amount of ice-cold isopropanol and centrifuge for 15 min at 12,000 g.
- Discard the supernatant and wash the pellet twice with 70% ethanol.
- Dry the pellet and dissolve it in 100 µl TE buffer.
- Add 10 µl of RNaseA (10 µg/ml) and incubate at 37ºC for 1 hr.
- Extract with equal volume of chloroform: isoamyl alcohol (24:1).
- Spin at 12,000 g for 10 min.
- Transfer the aqueous layer to a fresh 1.5 ml microfuge tube and add 2 volumes of ice-cold ethanol.
- Spin at 12,000 g for 5 min at room temperature.
- Wash pellet with 70% ethanol.
- Dry the pellet in vacuum and dissolve in 100 µl of TE buffer or sterile double distilled water and store at -20ºC until use.
- The DNA content can be checked by running on 0.7% agarose gel or by taking absorbance at 260 nm.
- Use about 2 µg DNA for restriction digestion and 25 ng for PCR amplification.

Restriction digestion

Genomic DNA (2-4 µg) was digested for 1 hr with 5 to 10 U of restriction enzyme under optimal temperature and buffer, following manufacturers recommendations (Fermentas, USA). The digested DNA fragments were fractionated on 0.7% agarose at 5 V/cm.

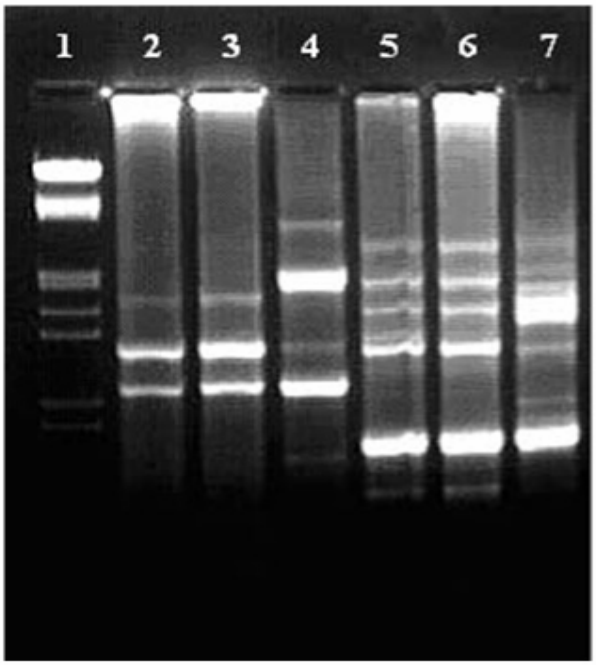

Figure 3. RAPD pattern of PCR amplified products on 1.2% agarose gel with OPA-20 primer from left to right. Lane 1: λ DNA digested with *Eco* R I and *Hind* III; lane 2: *Terminalia arjuna* fresh leaves; lane 3: *Terminalia arjuna* dry leaves; lane 4: *Terminalia bellerica* fresh leaves; lane 5: *Terminalia tomentosa* fresh leaves; lane 6: *Terminalia tomentosa* dry leaves; lane 7:*Terminalia chebula* fresh leaves.

PCR amplification

PCR was carried out in 25 µl volume reaction mixture. The reaction mixture contained 25 ng of DNA, 2.5 U *Taq* DNA polymerase enzyme (Fermentas, USA), 100 mM each dNTP, 2.5mM MgCl2, 1X *Taq* DNA polymerase buffer and 10 mM decamer primer (Operon, USA). The plant DNA was amplified using the following conditions with initial denaturation at 94ºC for 5 min, followed by 45 cycles, denaturation at 94ºC for 1 min, annealing at 38ºC for 1 min,

and extension at 72ºC for 3 min and final extension at 72ºC for 7 min. The PCR products were fractionated on 1.2% agarose gel using 1X TAE buffer containing 5 µg/ml ethidium bromide. Double digested λ DNA (*Eco* R I + Hind III) (Fermentas, USA) as molecular marker.

RESULTS AND DISCUSSION

The secondary metabolites produced by some plants possess important medicinal properties and are used in food, pharmaceutical, cosmetics and pesticide industries (Khanuja et al. 1999). *T. arjuna* contains ellagic acid, gallic acid, arjunic acid, arjungenin and their glucosides, arjunetin and arjunglucoside I (Bajpai et al. 2005; Pawar and Bhutani, 2005). The isolation of genomic DNA from perennial plants like *T. arjuna* is difficult due to presence of polyphenols and polysaccharides. During isolation procedure polysaccharides are found to form complexes with nucleic acids forming a gelatinous mass, thereby physically inhibiting the DNA from the action of DNA modifying enzymes *e.g.* restriction enzymes, DNA polymerase, ligase, etc. (Sharma et al. 2002). The polyphenols isolated along with DNA from *T. arjuna* are converted to several products reacting with proteins, and bring about their oxidation (Loomis, 1974). For the isolation of DNA from species like *T. arjuna* and *T. tomentosa*, we tried several published protocols like CTAB based (Doyle and Doyle, 1990), high salt and PVP (Porebski et al. 1997), high salt and sarcosyl (Sharma et al. 2002), combination of CTAB and SDS (Keb-llanes et al. 2002), using glucose as a reducing agent in standard CTAB protocol (Permingeat et al. 1998), and protocol for other species of *Terminalia* (Warude et al. 2003). The DNA isolated by above-mentioned methods was sticky, viscous and colored inhibiting the activity of DNA modifying enzymes. To overcome the problem of contaminant homogenization process. We crushed leaves in liquid nitrogen and removed the polyphenols by repeatedly washing 4-5 times using PVP and β-mercaptoethanol. The polysaccharides were removed using extraction buffer containing high NaCl concentration. Current protocol yields DNA of high purity and free from polyphenols and polysaccharides from fresh as well as dry leaves of *T. arjuna*, and other *Terminalia* species (Figure 1). The purity of the DNA sample was confirmed through its A_{260}/A_{280} ratio (1.8) and digestion with restriction enzyme *Eco*RI (Figure 2). On amplification using random amplification of polymorphic DNA (RAPD) primer the isolated DNA shows high intensity bands (Figure 3). In previously reported protocols for isolation of DNA from *T. arjuna*, commercial DNA isolation kit was used (Sarwat et al. 2006). Our method is simple and can be worked out using in -house-compounds, we tried modified protocols with higher concentration of CTAB (Khanuja et al. 1999). High ionic strength of CTAB forms complexes with proteins and most of the acidic polysaccharides (Jones and Walker, 1963); whereas high concentration of NaCl helps in the removal of polysaccharides (Aljanabi et al. 1999). In our study, we used the different concentrations of CTAB and PVP. However, in all the cases, low concentrations of polyphenols and polysaccharides were present at the end (data not shown). In our protocol, the polyphenols find their way in DNA preparation during the liquid nitrogen prepared reagents and is useful to extract DNA from fresh and dry leaves of *Terminalia* species.

In conclusion, the current method is simple and reliable for the isolation of genomic DNA from fresh and dry leaves of *T. arjuna*, which is known to be one of the complicated species for the isolation of DNA, due to the presence of a high percentage of secondary metabolites. This method was successfully applied for the isolation of DNA from other species of *Terminalia* like *T. bellerica*, *T. chebula*, and *T. tomentosa*.

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