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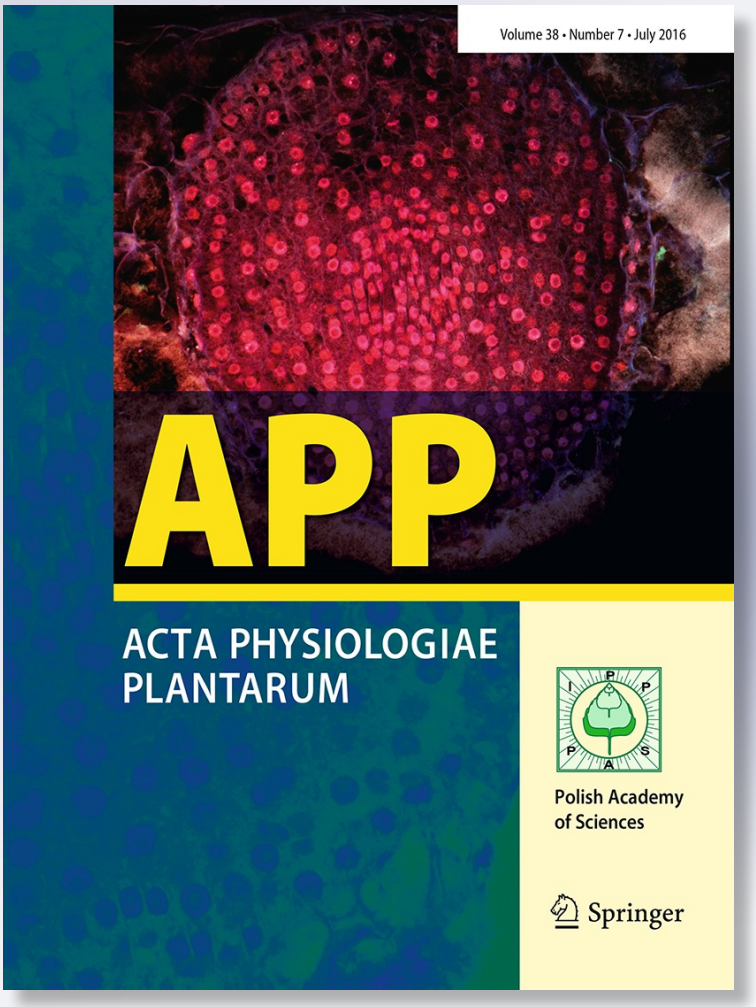

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SHORT COMMUNICATION

An improved method of DNA purification from secondary metabolites rich medicinal plants using certain chaotropic agents

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Abstract The presented work describes good quality DNA isolation method from mature leaves of some medicinally important plant species, viz. *Asparagus racemosus*, *Withania somnifera*, *Abrus precatorius*, *Commiphora wightii* and *Carissa carandas*. These plants hold immense medicinal values due to presence of certain secondary metabolites like polyphenols, terpenes, flavonoids, alkaloids, gums, resins, etc. Although these metabolites are accountable for important medicinal properties and authorize these plants to precedence over others, the same compounds disappoint the researcher while isolating high quality DNA. To overcome this problem, we propose a simple method in which DNA is adroitly bounded to diatomaceous earth in a solution of different chaotropic agent and alienated from intrusive compounds. Presented method affirms that secondary products, along with polysaccharides and proteins, can be perceptibly reduced by using silica matrix along with chaotropic agents. The described method is fast, simple and highly reliable for the isolation of DNA from obstinate plant species.

Keywords DNA isolation · Medicinal plants · CTAB · Chaotropic agents · Diatomaceous earth

Introduction

Medicinal plants are drawing more attention among modern day plant researchers since these are recognized as treasure box of future medicines. Alkaloids, polyphenols, polysaccharides, and other secondary metabolites, which are present in unusually high amount, are accountable for the medicinal properties of these plants. Use of molecular biology techniques would not only boost and make easy the production of these substances but also will pave new avenues in drug discoveries. Nevertheless, the same principle compounds which make these medicinal plants laudable of such intensive studies also hold back molecular approaches by creating problems at the time of isolation of good quality DNA. To initiate with molecular techniques, good quality DNA is considered to be foremost and very fundamental requirement. Leaf is considered to be the best suitable material for isolation of DNA but with mellowness, secondary products and polysaccharides content increases in leaves. These secondary products and polysaccharides co-precipitate along with DNA during procedural steps and create problems. Another considerable problem is that it is not always feasible to collect young and juvenile leaves from these medicinal plants throughout the year. Some medicinal plants like *Commiphora wightii* are deciduous and for most of the year remains without leaves. Likewise some endangered medicinal plants are restricted to specific unapproachable habitats and also the time of sample collection may not always coincide with period of active shoot development. To surmount all these problems, especially in medicinal plants, several modifications have been proposed in the routine CTAB method of DNA isolation which was originally described by Murray and Thompson (1980). These changes were focused on use of small initial amount of leaf

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material, juvenile tissue, polyvinylpyrrolidone (PVP), activated charcoal or hydrated ether, in pursuance of removing such intrusive compounds from subsequent extraction steps (Maliyakal 1992; Bi et al. 1996; Peterson et al. 1997; Porebski et al. 1997; Hernández et al. 2001; Martellossi et al. 2005; Kim et al. 2006; Haque et al. 2008; Ahmed et al. 2009; Amani et al. 2011; Echevarría-Zomeño et al. 2012).

In the course of our research on 'Molecular characterization of medicinal plants of western India', we encountered problems while isolating good quality DNA from medicinal plants particularly with *A. racemosus, W. somnifera, A. precatorius, C. wightii* and *C. carandas.* All these plants are having immense medicinal properties and exhibit diverse nature. The leaf characteristics, nature of plant, medicinal properties and secondary metabolite contents of these plants have been listed in Table 1. *Commiphora wightii* contain high amount of polyphenols, polysaccharides and other secondary metabolites like oleo–gum–resin. Oleo–gum–resin is a mixture of resin, gum and volatile oil and found in balsam canals of leaves of *C. wightii.* Oleo–gum–resin, along with the other products, forms complex with nucleic acid which is insoluble in buffer and create problems in further extraction steps (Guillemaut and Maréchal-Drouard 1992; Kumar and Shankar 1982). In *W. somnifera* more than 40 withanolides, 12 alkaloids and several sitoindosides have been reported from roots, berries and aerial parts. Withanolides, the chief secondary product (constituting 0.001–0.5 % dry weight) of this plant are restricted in leaves and cause problems in DNA isolation (Mirjalili et al. 2009). Similarly, other secondary products have also been reported from leaves and other parts of *A. precatorius* (Choi et al. 1989), *C. carandas* (Siddiqui et al. 2003) and *A. racemosus* (Negi et al. 2010) (Fig. 1).

The protocol described here is mainly developed for DNA extraction from mature leaf tissue of above mentioned five medicinal plants, known for their high secondary metabolite content. The extraction procedure is based on ability of different chaotropic agents (viz., $NaClO_4$, NaI and NaBr) to breakdown the hydrogen bonding in macromolecules like DNA and proteins, followed by specific binding and retention of nucleic acid on silica matrix, such as diatomaceous earth, in the shadow of these agents. The main role of chaotropic salts is in creating a hydrophobic environment, under which the silica membrane of the diatomaceous earth is the most suitable and available binding partner for the nucleic acids. Secondary metabolites, proteins and other contaminants do not bind to the silica membrane and are therefore washed out during the subsequent steps. Schematic diagram of principle steps has been depicted in Fig. 2.

Materials and methods

Plant material

Mature leaves were chosen for DNA isolation and were collected from wild and identified populations of *Asparagus racemosus, Withania somnifera, Abrus precatorius, Commiphora wightii* and *Carissa carandas.* Rice seedlings were used as a control to check the applicability of these modifications in relatively simple and easy to work with plants. After collection, leaves were bought to laboratory in ice and stored at −20 °C till further processing. The leaves were subjected to DNA isolation using two already established and routinely used protocols, i.e, CTAB method of Saghai-Maroof et al. (1984) and SDS method of Dellaporta et al. (1983). We have developed three alternative protocols in our laboratory with major modifications in original CTAB method.

DNA isolation

For total genomic DNA extraction, 1 g leaf tissue was crushed in mortar and pestle to a fine powder using liquid nitrogen and transferred to 50 mL polypropylene centrifuge tube containing 10 mL of CTAB buffer. Composition of CTAB buffer was maintained as per the description of Murray and Thompson (1980) i.e, 100 mM Tris (pH 8.0), 1.4 M NaCl, 20 mM Na_2EDTA (pH 8.0), 2 % CTAB (w/v) and 10 mM β–mercaptoethanol. 2 mM LiCl was added to this homogenate, and the mixture was incubated at 60 °C for 45 min followed by 10 min at room temperature. To this, Chloroform: isoamyl alcohol mix (in the ratio of 24:1) was added in equal volume. Centrifugation ($6000\times g$ at room temperature) was carried out and the upper aqueous phase was collected in a fresh tube. To it was added, ½ volume of 5 M NaCl and 1/10 volume of 3 M sodium acetate (pH 5.2). Chilled isopropanol was used to precipitate the DNA and centrifugation ($6000\times g$ for 10 min) was carried out to obtain the pellet. After two washes with 70 % ethanol, crude DNA pellet was allowed to dry and then suspended in 1 mL of TE buffer for dissolution.

Each 1 mL of crude nucleic acid extract was treated with three different binding buffers in three different experimental protocols. In protocol 1, one ml of nucleic acid extract was mixed with 3 volume of binding buffer 'A' [6 M $NaClO_4$, 50 mM Tris (pH 7.5) and 1 mM Na_2EDTA]. In protocol 2 and 3, binding buffer 'B' and 'C' were used, respectively, which were differing only in type of chaotropic agent used. In binding buffer 'B' and 'C', $NaClO_4$ was replaced with equimolar concentration of NaBr and NaI, respectively. After addition of binding buffer, the

Table 1 Leaf morphology, nature, secondary metabolite content and medicinal uses of the plants under study

Sr. no	Name of plant with chromosome number	Nature of plant	Leaf size, shape and type	Arrangement of leaf on stem, venation of leaf, division of leaf blade	Leaf margin, leaf surface, hairiness	Secondary metabolite reported	Medicinal values and plant part used in treatment
1	*Withania somnifera* ($2n = 48$)	Upright and stout shrub with central stem.	Up to 10 cm long and up to 5 cm wide, ovate, simple	Alternate, reticulate, leaf blade is ovate or oblong	Entire to sinuate, glabrous, whitish stellate-hairy, later becoming sparsely hairy	Alkaloids viz., cuscohygrine, anahygrine, tropine, pseudotropine, anaferine, isopellaterine, trophyltigloate. withanon, withaferin A, withanolides and withasomidienone (Kumar and Shankar 1982)	Medicinal properties—anti-stress, adaptogenic, aphrodisiac, sedative, diuretic, antispasmodic, germicidal, anti-inflammatory, nervine tonic. It enhances immunity and thyroid activity. Also is a good hypnotic in alcoholism (Khare 2008) Plant part used—root
2	*Carissa carandas* ($2n = 22$)	Large evergreen shrub with short stem	4.5–7 cm × 2.5–4 cm, ovate, simple	Opposite, reticulate, leaf blade is elliptic ovate	Entire, glabrous and shining, hairless	Alkaloids, saponin, glycosides, flavonoids, triterpenoids, phenolics, cardenolides and carbohydrates including starch and gums (Siddiqui et al. 2003)	Medicinal properties—used in ascariasis, biliousness, fever, internal bleeding. Rich source of iron and vitamin C so also used in anemia and scurvy (Devmurari et al. 2009) Plant part used—fruit
3	*Abrus precatorius* ($2n = 22$)	Slender, perennial climber	Oblong and rounded tips (i.e. obtuse apices) 2–5 inches long compound pinnate with 5–17 pairs of leaflets	Alternate, paripinnate, leaf blade is ovate or oblong	Entire, glabrous, hairless	Alkaloids viz. abrine, trigonelline, abruslactone A, hemiphloin, precatorine, glycyrrhizin, hypaphorine, abrusosides. Sugars viz. arabinose, galactose, xylose. montanyl alcohol, inositol, D monomethyl ether, pinitol. (Garaniya and Bapodra 2014)	Medicinal properties—anti diabetic, anti migraine, antiserotonergic, antispasmodic, bronchodilator, antitumor, aphrodisiac, larvicidal activity, neuromuscular blocking activity, antibacterial activity (Khare 2008) Plant part used—fruit and leaves
4	*Asparagus racemosus* ($2n = 20$)	Woody climber	Pine needle, 1–2 cm long	Pine needle like phylloclade, spiky stem	Needle like, shiny surface, hairless	Saponins, sitosterol shatavaroside, asparagamine, polycyclic alkaloid. Alcohol viz. acemofuranborneol, myrtanol, pinocarveol, ethylhexanol. Aldehydes viz. perillaldehyde, hydroxyethylbenzaldehyde (Negi et al. 2010)	Medicinal properties—prevent aging, increase longevity, impart immunity, brain tonic, vigor and add vitality to the body and it is also used in nervous disorders, dyspepsia, inflammation, neuropathy, hepatopathy, antiulcer, antioxidant, and antidiarrhoeal, antidiabetic (Alok et al. 2013) Plant part used—root
5	*Commiphora wightii* ($2n = 26$)	Woody shrub	1–5 cm long, 0.5–2.5 cm broad, ovate, simple or trifoliate	Simple trifoliate leaves with ovate margin	Entire, shinning, green	Resins, diterpene hydrocarbon, diterpene alcohol, guggulsterone, sesamin and camphorene. (Kulloli and Kumar 2013)	Medicinal properties—anti-arthritis, inflammation, bone-fractures, obesity and disorders of lipid metabolism (Kulloli and Kumar 2013) Plant part used—stem, bark, leaves

mixture was allowed to settle for 30 min and then subsequently centrifuged at 550×*g* for 10 min. The supernatant was collected in a fresh polypropylene centrifuge tube. Separately prepared diatomite suspension (300 µL) was added to this supernatant and contents were mixed for 30 min by regular gentle inversion to permit the binding of DNA and diatomite. The tubes were then centrifuged at 550×*g* for 10 min and supernatant was discarded. The pelleted DNA/diatomite complex was re-suspended in 1.5 mL of wash buffer 1 (binding buffer and distilled water in the ration of 3:1) and centrifuged at 3000×*g* for 15 s. The pellet was re-suspended in 1.5 mL of Wash buffer 2 and again centrifuged at 3000×*g* for 15 s. Wash buffer 2 was prepared by mixing equal volume of mix 'a' [40 mM Tris (pH 8.0), 0.8 M NaCl and 4 mM Na_2EDTA] and absolute ethanol. The supernatant was discarded carefully and the DNA-diatomite pellet was allowed to dry completely. Finally it was suspended in 300 µL TE buffer (10 mM Tris, pH 8.0, 1 mM Na_2EDTA) to dissolve. The tubes were kept on water bath at 40–50 °C for 20–30 min, intermittent inversion was done for proper dissolution of DNA in TE buffer, and finally tubes were centrifuged (11600×*g* for 60 s) to discard the undissolved diatomite particles. The upper supernatant phase of TE buffer (containing dissolved genomic DNA) was collected in a fresh tubes and to it was added 100 µL 1 M NaCl and 300 µL isopropanol for precipitation of DNA. The tubes were kept at −20 °C for 2 h before centrifugation at 11,600×*g* for 5 min. The supernatant was discarded and the pellet was allowed to dry completely. Finally the pellet was dissolved in 100 µL of 0.1× TE buffer and stored at −20 °C.

Fig. 1 Leaf morphology of plants under study *1 C. wightii*, *2 C. carandas*, *3 A. precatorius* (with seedpod), *4 A. racemosus* and *5 W. somnifera*

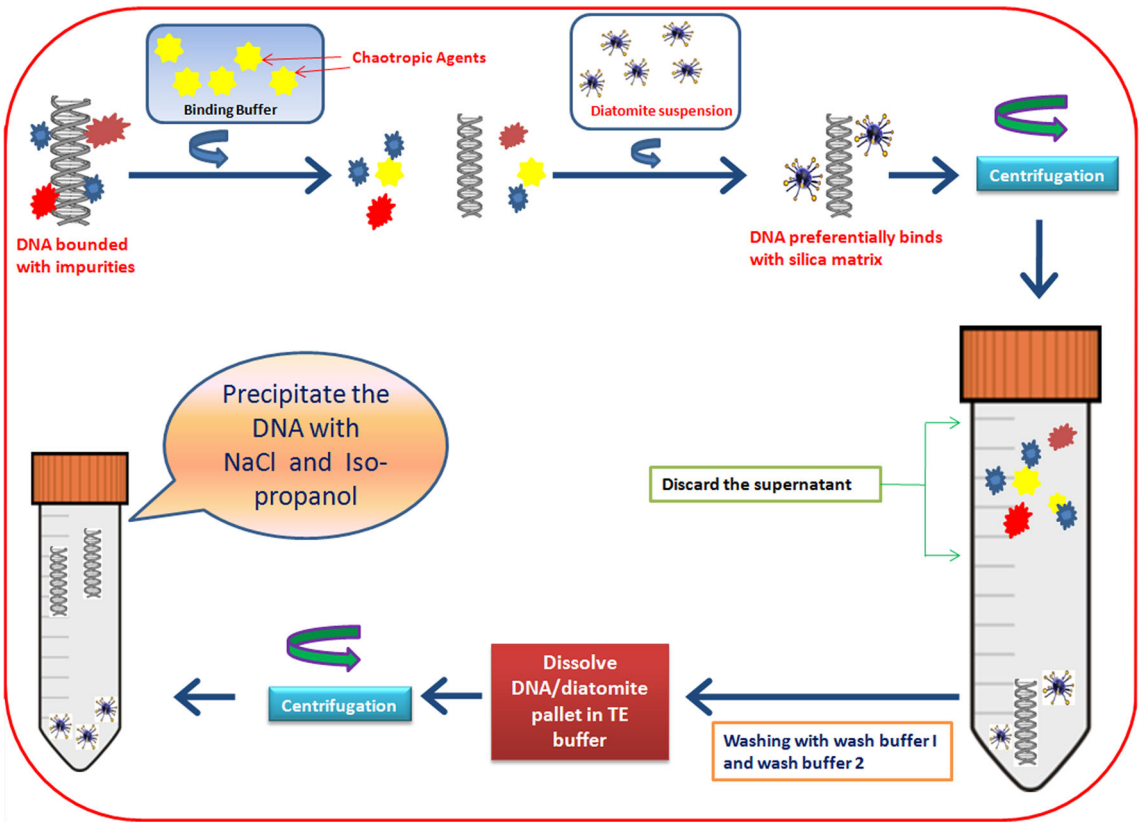

Fig. 2 Schematic diagram of principle steps involved in purification of DNA using modified protocol

All the experiments were repeated six times to check the reproducibility and consistency of the results.

Preparation of diatomite suspension

Diatomaceous earth solution was prepared as per the method described by Kim and Pallaghy (1996). The diatomaceous earth (HiMedia Labs) was blended (total 50 mL with concentration of 50 mg/mL) in deionized distilled water using mortar and pestle and allowed to settle down for 3 h without any disturbance. White milky supernatant containing very fine particles was carefully thrown away. This process was repeated at least 3–4 times. The washed diatomite was collected by centrifugation at low RCF i.e. $112\times g$ for 5 min. The intact sediment of diatomite was then re-suspended in original volume of deionized distilled water and autoclaved. Working stocks were prepared and stored at 4 °C. Vortexing just prior to use of the solution is suggested to build uniform suspension.

Gel electrophoresis

The DNA was run on agarose gel to check its quality. 4.0 µl of DNA sample was mixed with 1.0 µl of 5 × DNA loading dye on a Parafilm^{TM} strip and mixed well with the help of a micropipette. DNA samples were subjected to electrophoresis in 1× TAE buffer for approximately 1 h at 80 V on 1 % agarose gel matrix. Photographs of gel were taken under UV light using a gel document system (Bio-Rad USA). All reactions were repetitively performed three times to confirm the result.

DNA quantification

Concentration of DNA was measured using UV–Vis Spectrophotometer (Pharmaspec UV-1800, Shimadzu, Japan). Absorbance of the solution was measured at wavelengths 260, 280 and 230 nm. The ratio of absorbance at A_{260}/A_{280} nm and A_{260}/A_{230} nm was also noted to check the purity of DNA samples.

Restriction digestion

Restriction digestion of isolated DNA samples was performed to determine the suitability of DNA for molecular studies. For this, 2 units of enzymes (viz., EcoR I, Hind III and Alu I, purchased from New England Biolabs. UK) were mixed with 2.5 µg of DNA samples and mixture was incubated overnight at 37 °C. After incubation, the digested DNA samples, along with undigested controls were subjected to electrophoresis in 0.8 % agarose gel.

Molecular screening of isolated DNA using PCR amplification

To confirm the suitability of isolated DNA for molecular studies, the isolated DNA from modified methods was subjected for RAPD analysis. After initial screening, a total of 5 arbitrary RAPD 10-mer primers were selected for amplification of genomic DNA in experimental and control plant species. RAPD amplification was carried out in a volume of 20 µl reaction mixture having 20 ng of genomic DNA, 1.5 mM dNTP (Invitrogen), 20 pmol of primer, 1 U of Taq polymerase, 2 mM $MgCl_2$ and 1 × PCR assay buffer. PCR amplification steps involved; initial denaturation at 94 °C for 4 min followed by 40 amplification cycles, each consisting of; (1) denaturation at 94 °C for 1 min, (2) annealing at 37 °C for 1 min and then (3) extension at 72 °C for 2 min. Final extension of the product was done for 7 min at 72 °C. All components used in PCR amplification were purchased from Merck (India). Amplification was carried out in a DNA thermal cycler (Applied biosystem, USA) and amplified products were resolved in 1.5 % agarose gel. Photographs of the gels were taken using gel documentation system (Bio-Rad, USA).

Result and discussion

In the current study, DNA was isolated from mature leaves of five different medicinal plant species in which high level of secondary metabolites has been reported. The new protocol was developed and optimized by making several changes in existing standard protocols. The quantity and quality of the initial raw complex of DNA, obtained at isopropanol precipitation stage of standard CTAB method, were highly inconsistent among the species tested. Only *W. somnifera* and *A. precatorius* produced clear product at this stage while in case of *C. wightii* and *C. carandas*, we obtained highly viscous and dirty yellow product which formed sticky pellet. The pellet dissolved barely in TE buffer and was observed as smear with a very faint band of genomic DNA on the agarose gel. The amount of DNA obtained using routine standard CTAB method (Saghai-Maroof et al. 1984) in *C. wightii* and *C. carandas* was 15 and 18 µg, respectively, for 1 g of leaf tissue (Table 2). In this method absorbance ratio ($A_{260/280}$) for both the plant species was recorded less than 1.3, which indicates very poor quality of DNA.

In the other routine standard method i.e. Dellaporta et al. (1983), CTAB was substituted by other anionic detergent SDS. This method also failed to recover high quality and quantity of DNA. In lieu of clear and non-sticky pellet, we obtained opaque and gelatinous product in *C. wightii* and *C. carandas* using this method. In case of *C. wightii*, it was

Table 2 Yield and purity of DNA obtained from *A. racemosus*, *W. somnifera*, *C. carandas*, *A. precatorius* and *C. wightii* using various protocols

Plant	Concentration (µg per g of fresh leaf tissue) and purity ($O.D_{260/280}$ and $O.D_{260/230}$) of DNA											
	Standard CTAB method (Saghai-Maroof et al. 1984)			Standard SDS method (Dellaporta et al. 1983)			Protocol 1 (using NaI as chaotrope)			Protocol 2 (using $NaClO_4$ as chaotrope)			Protocol 3 (using NaBr as chaotrope)		
	DNA Conc.	$O.D_{260/280}$	$O.D_{260/230}$	DNA Conc.	$O.D_{260/280}$	$O.D_{260/230}$	DNA Conc.	$O.D_{260/280}$	$O.D_{260/230}$	DNA Conc.	$O.D_{260/280}$	$O.D_{260/230}$	DNA Conc.	$O.D_{260/280}$	$O.D_{260/230}$
Asparagus racemosus	60.0 ± 2.0	1.54 ± 0.01	1.33 ± 0.08	40.0 ± 1.9	1.72 ± 0.02	1.22 ± 0.16	42.0 ± 1.7	1.64 ± 0.05	1.72 ± 0.11	40.0 ± 2.4	1.68 ± 0.03	1.75 ± 0.54	24.9 ± 2.3	1.74 ± 0.05	1.92 ± 0.07
Withania somnifera	50.2 ± 2.1	1.78 ± 0.02	1.67 ± 0.14	95.0 ± 1.4	1.65 ± 0.01	1.69 ± 0.03	65.0 ± 1.0	1.73 ± 0.06	1.88 ± 0.41	75.0 ± 2.9	2.09 ± 0.10	1.99 ± 0.21	28.0 ± 2.2	1.63 ± 0.05	1.78 ± 0.20
Carissa carandas	18.0 ± 2.1	1.32 ± 0.02	1.42 ± 0.23	135.0 ± 3.0	1.25 ± 0.03	1.39 ± 0.25	45.0 ± 1.7	1.67 ± 0.07	1.81 ± 0.66	43.0 ± 2.3	1.86 ± 0.04	1.91 ± 0.44	34.9 ± 2.0	1.69 ± 0.03	1.81 ± 0.02
Abrus precatorius	65.0 ± 1.2	1.66 ± 0.03	1.84 ± 0.17	115.0 ± 2.4	1.78 ± 0.04	1.84 ± 0.09	70.0 ± 2.68	1.94 ± 0.05	2.10 ± 0.71	60.0 ± 2.8	1.81 ± 0.05	2.08 ± 0.11	85.0 ± 2.4	1.84 ± 0.08	1.98 ± 0.12
Commiphora wightii	15.1 ± 1.2	1.21 ± 0.10	1.33 ± 0.14	12.0 ± 2.0	1.33 ± 0.04	1.25 ± 0.36	30.0 ± 2.25	1.62 ± 0.03	1.80 ± 0.41	44.9 ± 1.75	1.72 ± 0.03	1.79 ± 0.29	37.0 ± 3.1	1.87 ± 0.07	1.97 ± 0.13
Rice seedlings (as a control measure)	136.3 ± 2.3	1.82 ± 0.12	1.94 ± 0.23	128.8 ± 4.3	1.77 ± 0.09	1.96 ± 0.23	98.0 ± 2.3	1.80 ± 0.12	1.98 ± 0.09	112.3 ± 2.3	1.79 ± 0.77	2.12 ± 0.07	92.0 ± 4.2	1.75 ± 0.23	1.98 ± 0.33

difficult to dissolve the DNA pellet in TE buffer, obtained after initial precipitation stage. The loaded sample stuck to wells of agarose gel during electrophoresis. Although quantity of DNA was recorded comparatively high in case of *C. carandas* (135 µg per g of fresh leaf tissue) but it was of poor quality as evident by very low A_{260}/A_{280} ratio (i.e. 1.25). Although this protocol proved good in terms of quality for *A. racemosus* and *A. precatorius* (A_{260}/A_{280} is 1.8) but again not considered as good deed cause the yield was recorded low (Table 2). The above two standard popular methods of DNA isolation gave better results in relatively simple to work with rice seedling (which in this case was taken as control plant), in terms of quantity and quality (Fig. 3).

As discussed in the introduction and mentioned in Table 1, the plants under the study are rich in secondary metabolites like alkaloids, polyphenols, polysaccharides, etc. In oxidized form these phenolics binds to protein and DNA, giving DNA yellow brown color. The viscosity and stickiness of the DNA is due to the presence of polysaccharides, gum and other secondary products (Angeles et al. 2005; Katterman and Shattuck 1983). This type of colored and viscous DNA preparation is useless for any further molecular studies.

Diatomaceous earth consists of fossilized remains of diatoms and is formed of intricately patterned silica shell of 10–200 µm and can be used in several purposes including purification of nucleic acid (Bozarth et al. 2009). Chaotropic agents (like $NaClO_4$, NaBr and NaI) increase the entropy in a solution. The proteins, secondary products, polysaccharides and other contaminants cause hydrophobic regions to come together and give rise to a pseudo-interaction between these molecules. The water molecule get arranged around these structure and create a protecting shell, which results into reduction of entropy and do not allow the DNA molecules to get precipitate out of the solution (Salvi et al. 2005). The chaotropic agents like

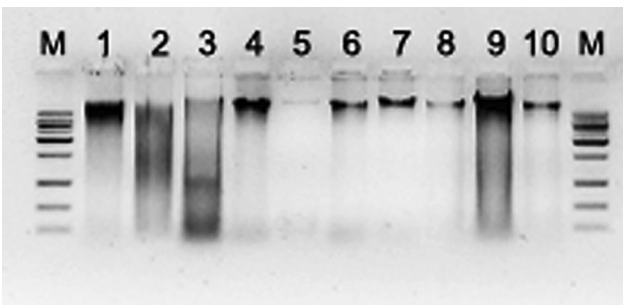

Fig. 3 Isolated DNA from standard SDS method of Dellaporta et al. 1983 (*lane 1–5*) and standard CTAB method of Saghai-Maroof et al. 1984 (*lane 6–10*) on 0.8 % agarose gel. The tested plant samples were *A. racemosus* (*lane 1* and *6*), *W. somnifera* (*lane 2* and *7*), *C. carandas* (*lane 3* and *8*), *A. precatorius* (*lane 4* and *9*) and *C. wightii* (*5* and *10*). *M* represent 1 kb DNA ladder

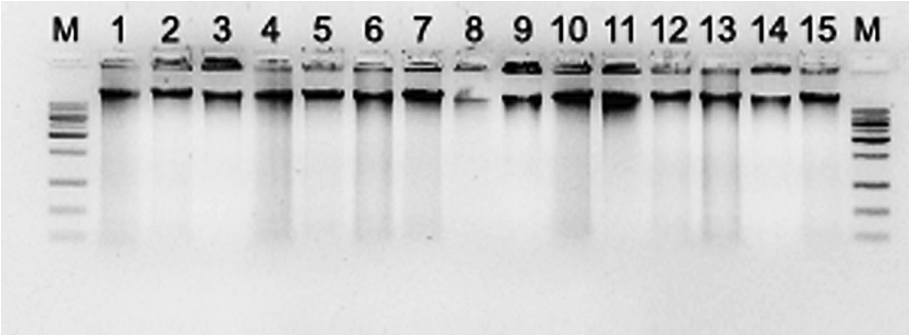

Fig. 4 Isolated DNA from Protocol 1 (*lane 1–5*; using $NaClO_4$ as chaotropic agent), Protocol 2 (*lane 2–10*; using NaBr as chaotropic agent) and Protocol 3 (*lane 11–15*; using NaI as chaotropic agent) were resolved on 0.8 % agarose gel. The tested plant samples were *A. racemosus* (*lane 1, 6* and *11*), *W. somnifera* (*lane 2, 7* and *12*), *A. precatorius* (*lane 3, 8* and *13*), *C. wightii* (*lane 4, 9* and *14*) and *C. carandas* (*5*, *10* and *15*). *M* represent 1 kb DNA ladder

$NaClO_4$ and Guanidium ions interact with these molecule and disrupt the water shell and disorder the stable arrangement between these molecules, hence the entropy of the system increases. In those footing circumstances, the silica particles are best suitable and available particles in the solution for binding with DNA molecules. The use of chaotropic agents in removal of proteins and alkaloids have been reported previously (Hatefi and Hanstein 1969 and Flieger 2007). Boom et al. (1990) has first of all reported that, in the presence of chaotropic agent and other solvent like Gdm^+-salts, nucleic acids has more affinity towards silica and hence DNA specifically adsorbed by silica particles and can be purified from human serum or urine. Gilmore et al. (1993) has reported selective binding of diatomaceous earth in the presence of $NaClO_4$ for purification of plant genomic DNA. The other substitute ions in Gdm-salt or $NaClO_4$, like Cl^-, Br^-, I^- and SCN^- also have large positive entropies which can be interpreted as strong structure breaking effect of such ions on water (Hatefi and Hanstein 1969). The potential of these chaotropic agents in the presence of silica particles has never been checked in isolation of DNA from medicinal plants. Vogelstein and Gillespie (1979) have used glass silica in the presence of NaI for purification of DNA from agarose gel after electrophoresis. These studies have encouraged us to try the potential of NaI, NaBr and $NaClO_4$ in association with diatomaceous earth for purification of nucleic acid from recalcitrant medicinal plants. Another hurdle in standard CTAB and SDS method of DNA isolation is contamination of RNA. Generally, RNA is removed through enzymatic treatment using RNase A and subsequent purification by chloroform/isoamyl alcohol treatment which generally lower the DNA yield. We preferred the use of LiCl in our protocol to precipitate the RNA, as it is already established that LiCl not only binds preferentially to RNA but also additionally remove the proteins and neutral polysaccharides (Ahmad et al. 2004).

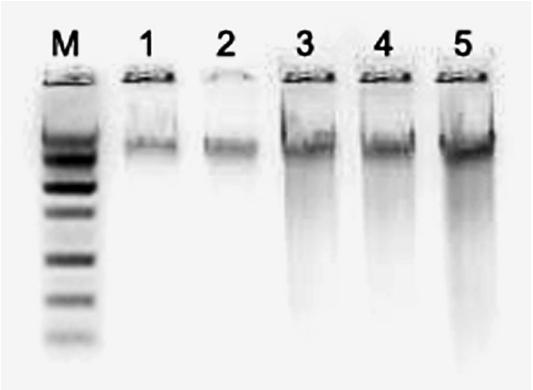

Fig. 5 Isolated DNA samples of rice seedling (control plant) from standard SDS method of Dellaporta et al. (1983) (*lane 1*) and standard CTAB method of Saghai-Maroof et al. (1984) (*lane 2*) along with modified Protocol 1(*lane 3*; using $NaClO_4$ as chaotropic agent), Protocol 2 (*lane 4*; using NaBr as chaotropic agent) and Protocol 3 (*lane 5*; using NaI as chaotropic agent) were resolved on 0.8 % agarose gel. *M* represent 1 kb DNA ladder

In the present study, we tried diatomaceous earth along with three different chaotropic agents for isolation and purification of DNA from five diverse recalcitrant medicinal plants as it is the known fact that a sole scrupulous DNA isolation method is not likely to be suitable for all plants (Loomis 1974). Chemotypic absurdity in different plants does not allow optimal and qualitative yield of DNA using a single method; therefore, even the species which are closely related, may sometime necessitate isolation protocols which are poles apart (Weishing et al. 1995). In marked contrast with the two standard routine methods, our protocols proved unanimously good in terms of quality as evident from A_{260}/A_{280} ratio of 1.6–2.0, irrespective of the chaotropic agent used. In case of *C. wightii* and *C. carandas*, where both the routine methods were found to be of substandard, our method raised the final yield of DNA up to 45 µg per gram of fresh tissue, with good quality as

well. In both the plants, A_{260}/A_{280} ratio was recorded 1.8 while we used $NaClO_4$ and NaI as chaotropic agent (Table 2). In case of *W. somnifera* protocol 2 (where $NaClO_4$ was used as chaotropic agent) proved best in terms of quantity and quality (75 µg per gram with $A_{260}/A_{280\ ratio}$ of 2.0). In case of *A. racemosus* all the three developed protocol yielded DNA concentration in the range of 25–40 µg per gram with absorbance ratio range in 1.6–1.7. The probable reason behind low DNA yield in some cases, compared with the standard method tried in other plant species, may be due to loss of DNA during washing of silica matrix/DNA complex with ethanol to remove chaotrope. The variation in final yield of DNA in the same test plant, when chaotropic agents is changed, may be due to disparity in potential of different chaotropic ions in breaking down the lipophilicity of water molecules. This explanation is in agreement with the conclusions of Hatefi and Hanstein (1969) regarding the possible structure breaking effect of such ions on water. Moreover, diverse plant species significantly differ in type and concentration of sugars and polyols, which considerably reduce the efficacy of chaotropic agents in denaturing contaminants

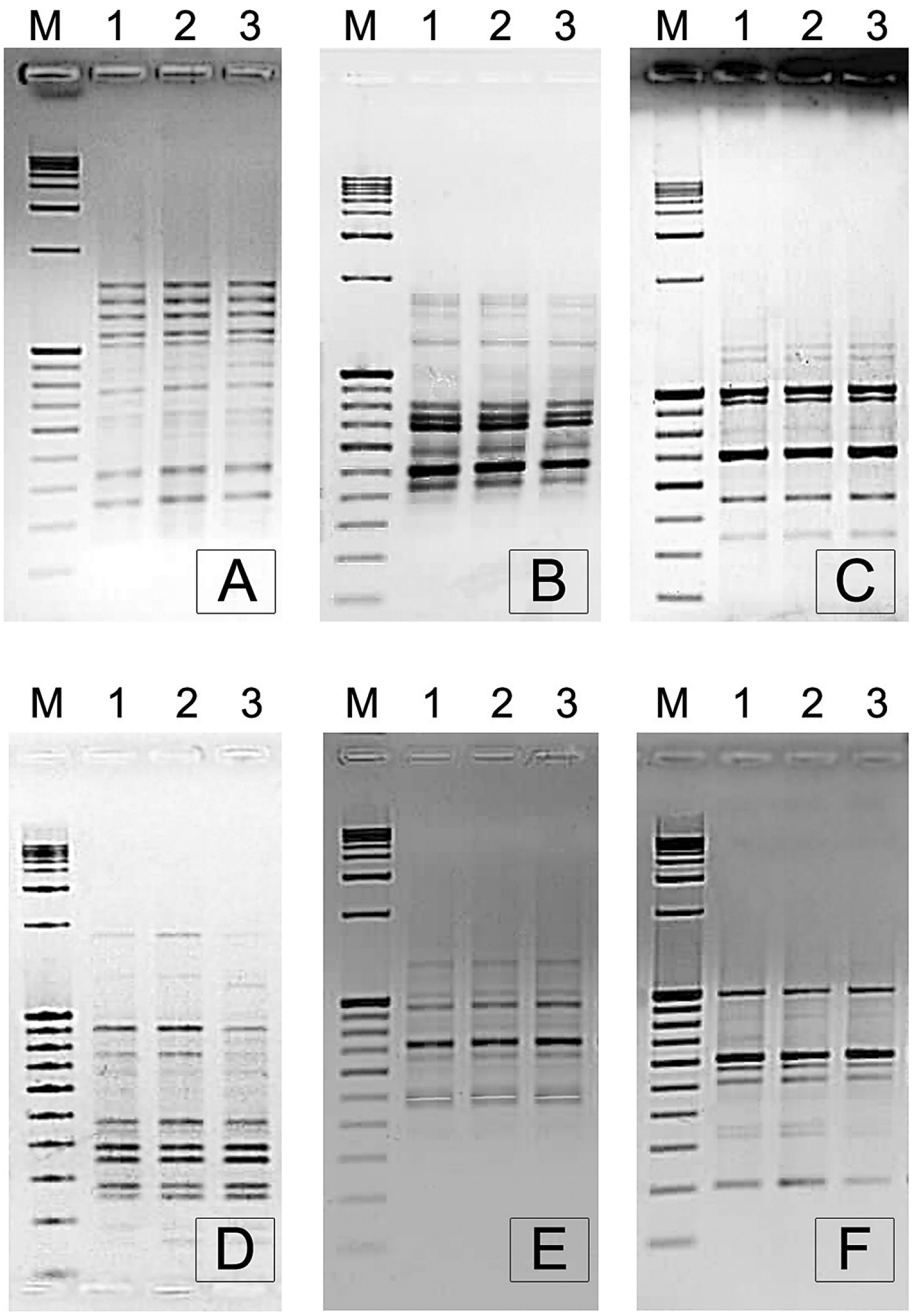

Fig. 6 PCR amplification of genomic DNA of different plants using RAPD primers. RAPD profile of **a** *A. racemosus*; generated using random decamer primer OPA 4 (sequence 5′–3′; AATCGGGCTG), **b** *W. somnifera*; generated using random decamer primer OPG 02 (sequence 5′–3′; GGCACTGAGG), **c** *C. carandas*; generated using random decamer primer OPA 10 (sequence 5′–3′; GTGATCGCAG), **d** *A. precatorius*; generated using random decamer primer OPC 01 (sequence 5′–3′; TTCGAGCCAG), **e** *C. wightii*; generated using random decamer primer OPK 16 (sequence 5′–3′; GAGCGTCGAA) and **f** rice seedling; generated using random decamer primer OPA 4 (sequence 5′–3′; AATCGGGCTG). *Lane 1*, *2* and *3* in all **a**–**f** represents amplification products using genomic DNA isolated from Protocol 1 ($NaClO_4$ as chaotropic agent), Protocol 2 (NaBr as chaotropic agent) and Protocol 3 (NaI as chaotropic agent)

(Taylor et al. 1995). In addition, it is established fact that different plants have different chromosome number, size and water content; as a result the DNA content per gram fresh weight will also change. Besides this, plants contain a variety of nucleases which degrade the long DNA strands into short pieces that do not precipitate well with alcohol. Since the type and content of nucleases changes from plant to plant, the final recovery of DNA will also change (Figs. 4, 5).

A_{260}/A_{230} ratio is another important parameter to check the suitability of DNA samples for molecular studies. Contaminant like alkaloids, carbohydrates, peptides, phenols or those other compounds which contain benzene ring, absorb the light at 230 nm. DNA samples having low A_{260}/A_{230} ratio (i.e, less than 2.0) is an indication of presence of such compounds that may interfere with other downstream process like PCR, microarray or gene expression (Echevarría-Zomeño et al. 2012). While standard CTAB or SDS method was adopted for DNA isolation, in all the five different plant species under the study (except *Abrus precatorius*), have shown significantly low A_{260}/A_{230} ratio. In all these plants, A_{260}/A_{230} ratio was found to be in ranges of 1.2–1.6 using these standard methods. This clearly indicates that the isolated DNA from these routine methods is not suitable for molecular studies in these plants. In contrast to this, the modification brought into play with all our three protocols results into high A_{260}/A_{230} ratio (ranging from 1.7 to 2.1) in all plants under study including the control rice seedlings. This evidently shows that the isolated DNA is good enough for molecular studies and moreover, the protocols are not restricted to only chemically complex plant species rather than can be applied to other simple and easy to work with plants. This was also confirmed by PCR amplification of genomic DNA of these species using random decamer primers. Figure 6 shows clear, scorable and highly reproducible band of genomic DNA obtained from RAPD analysis. The unimpeachable nature of DNA was reaffirmed through complete digestion of samples by the restriction enzyme EcoRI (Fig. 7), HindIII and AluI (data not shown). This clearly shows that the isolated DNA is biddable to further applications in cloning and other molecular experiments like fingerprinting.

The supremacy of our method, when weighed up with other routine SDS and CTAB method, is that flawless and pukka DNA can be obtained even from mature leaves of gum yielding and highly recalcitrant medicinal plants whereas the routine methods do not make any grade.

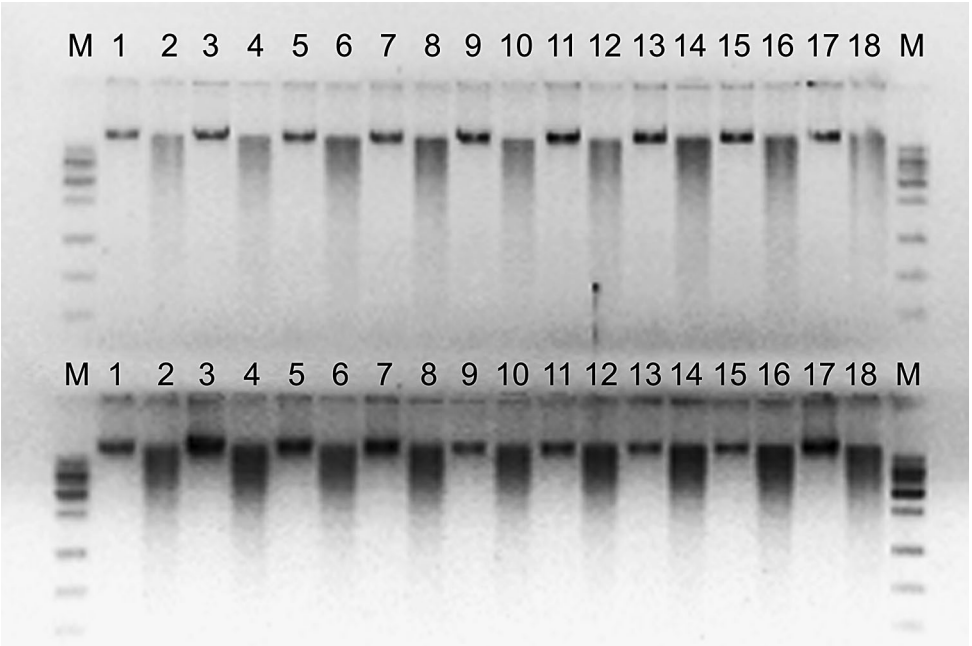

Fig. 7 Restriction endonuclease digestion pattern (digested with EcoRI) of genomic DNA isolated using Protocol 1 (*upper lane 1–12*; using $NaClO_4$ as chaotropic agent), Protocol 2 (*upper lane 13–18* and *lower lane 1–6*; using NaBr as chaotropic agent) and Protocol 3 (*lower lane 7–18*; using NaI as chaotropic agent). The undigested and digested DNA samples were of *A. racemosus* (undigested DNA; *upper lane 1*, *13* and *lower lane 7*, digested DNA *upper lane 2*, *14* and *lower lane 8*), *W. somnifera* (undigested DNA; *upper lane 3*, *15* and *lower lane 9*, digested DNA *upper lane 4*, *16* and *lower lane 10*), *A. precatorius* (undigested DNA; *upper lane 5*, *17* and *lower lane 11*, digested DNA *upper lane 6*, *18* and *lower lane 12*), *C. wightii* (undigested DNA; *upper lane 7* and *lower lane 1* and *13*, digested DNA *upper lane 8*, *lower lane 2* and *14*), *C. carandas* (undigested DNA; *upper lane 9* and *lower lane 3* and *15*, digested DNA; *upper lane 10* and *lower lane 4* and *16*) and rice seedling (undigested DNA; *upper lane 11* and *lower lane 5* and *17*, digested DNA; *upper lane 12* and *lower lane 6* and *18*). *M* represent 1 kb DNA ladder

Moreover, our method is quicker as compared to the routine SDS and CTAB method, where extra time taking steps of RNAse treatment for removal of RNA contamination and then subsequent re-precipitation, washing and drying of DNA pellet are involved.

In conclusion, the novel method of isolation and purification of DNA, described here, is quick, simple and most reliable for obstinate plant species and can be adopted as standard method for other similar plants where DNA isolation is a tuff task.

Author contribution statement Dr. Preetam Joshi: conceptualization and designing of protocol and experimentation; Dr. Anil Kumar Singh: critical review of work and experimentation; Miss Bansi Ghadia: experimentation (Protocol development); Miss Mansi Hirpara: experimentation (Protocol development); Miss Tannu Khatnani: experimentation (PCR analysis and restriction digestion); Dr. Shivani Patel: manuscript writing and editing.

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