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A MODIFIED CTAB METHOD FOR DNA EXTRACTION OF OILY PLANTS OF BRASSICACEAE FAMILY

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Abstract

Extraction of DNA from secondary metabolite enriched medicinal plants has always been tricky. Numerous methods for DNA extraction has been presented but those either gives poor yield or employed to least chemical heterogenetic plants species with use of costly and hazardous chemicals and conditions that become worse for oily plants. To our knowledge only a few protocols have been reported for DNA extraction from oily plants that not only are laborious but also involve very complex reagents. Here we report simple DNA extraction strategy comparable to costly commercial DNA extraction kits which is equally potent for oily and non-oily plants. High-molecular weight DNA was extracted without the use of liquid nitrogen and RNase A treatment that markedly reduce the extraction cost. Quantity of the DNA extracted from species with lower nuclear DNA content was in the range of 250-380 µg/mL with A_{260}/A_{280} absorbance ratio 1.67-1.87 that confirmed purity of the recollected DNA samples. Quality of the extracted DNA was also evaluated by restriction analysis and PCR amplification. This protocol gives DNA that is suitable for molecular biology techniques sensitive to contaminants.

Method Summary

We developed a short CTAB based methodology with two unique extraction buffers to sieve out high quality DNA in good quantity from oil enriched medicinal plants.

Keywords: Nuclear DNA, Oily Plants, Brassicacea, CTAB (Cetyl Trimethyl Ammonium Bromide), A_{260}/A_{280} Absorbance, RNaseA.

INTRODUCTION

Extraction of deoxyribonucleic acid (DNA) from biological samples is one of the foremost step to make headways in molecular biology. DNA extraction from medicinal and ornamental plants has been a hideous process mainly as these plant species are gifted with numerous secondary metabolites such as flavonoids, gummy polysaccharides, terpenes, alkaloids and phenols (1). Undoubtedly, significance of medicinal plants for cosmetic, pharmaceutics and food industries etc mostly reside in these compounds (2, 3). The metabolites and proteins form complexes with eukaryotic DNA and deteriorate

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the yield and quality of high molecular weight extracted DNA (4). Moreover, DNA degradation by nucleases, co-precipitation of polyphenols, viscous polysaccharides and other inhibitory compounds that hamper down streaming reactions directly/indirectly are the main problems confronted during DNA extraction procedures (5, 6). Hence, a method to get pure and high throughput DNA is high in need. Commercially available nucleic acid extraction kits are not usually advocated due to constrains like high cost, effective for only limited number of samples and generates large quantity of waste (7). Manual method is preferred in many cases as these give customized solutions in various difficult samples.

Cationic detergent CTAB based protocol has been reported to yield good quality DNA as it promotes the eukaryotic cell lysis *in situ* by hydrolyzing the ionic interaction of phospholipid bilayer and remove most of the polysaccharides and polyphenols without involving any hazardous chemical (6, 8).since then a variety of nucleic acid extraction procedures based on CTAB has been devised. However, these procedures only work for a limited number of plant species or tissues. Moreover, a single protocol is not sufficient to extract the DNA from chemotypic heterogenetic plants species as they dissent optimal DNA yield even different strategies are needed for akin species (6, 9).

The present protocol is devised to recover utmost amount of DNA from heterogeneous plant tissues rich in secondary metabolites restraining the co-extraction of inhibiting substances. Plants used in this study belong to Brassicaceae family having lower content of nuclear DNA (10). These plant species also have much higher content of oil along with other abundant polysaccharide and polyphenols contaminants. This trace amount of DNA is tangled in oil and polysaccharide-polyphenol mesh and cannot be eluted. Protocols reported in literature did not work well for these difficult plants species. Hence in order to overcome these drawbacks, we followed the doublet approach for detergents and antioxidants that not only speeded up the lysis reaction but also captured other contaminants and not let those to bind DNA. In this study we have skipped the use of liquid nitrogen, hydrolyzing enzymes and polysaccharide binding resins, thus reducing the cost of the protocol. These modifications has tailored an efficient, simple and cost-effective nuclear DNA extraction protocol for difficult plants species. To increase its validity the method was also tested for some plant species belonging to Fabaceae and Asteraceae that yielded satisfactory results.

MATERIALS AND METHODS

Collection of Plants

Chemically heterogenic Plant species rich in secondary metabolites, different families were collected: *Cirsium arvense, Sonchus arvensis, Brassica compestris, Eruca sativa, Brassica juncea, Brassica nigra, Brassica napus, Alhagi marourum* and *Acacia modesta.* Each plant sample comprised three replicates. All specimens were collected from their natural habitat and weighed. Parts of the plants were ensured to be free from any disease. Only leaf and root portion of the plants were used. Plants were washed carefully and completely with distilled water and air dried at room temperature before DNA extraction.

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A direct relationship between weight of tissue and yield of DNA has been depicted (15) but here we followed the strategy of identical weight for all plant tissue samples.

DNA Extraction Protocol

Each plant sample was cut into small pieces with sterilized scissors and about 3 × 200 mg was taken into a mortar. Tissues were crushed with pestle in the presence of 3 × 350 µL DNA extraction buffer 1 {2 M NaCl (Merk), 70 mM EDTA (Bio-Rad), 0.25% triton-X100 (Sigma), 0.2 M DIECA (Merk), 0.4 M ascorbic acid and 3.5% CTAB (Bio-Rad)} till the tissue was converted to a foggy dark green paste. 1% (w/v) PVPP (EMD) and 1% (v/v) PVP40 (Sigma-Aldrich) were added directly to the plant tissue along with extraction buffer. Then 3 × 300 µL pre-warmed extraction buffer 1 was added further and mixed thoroughly. The paste was poured in microcentrifuge tubes and incubated for 20 minutes at 65 ºC followed by incubation in an incubator shaker for 3 minutes at 200 rpm. The tubes were incubated again at 37 ºC in a dry bath for 10 minutes and then centrifuged at 120000 rpm for 10 minutes. Supernatant was extracted in 400 µL of DNA extraction buffer 2 {100 mM Tris-Cl, 50 mM EDTA, 1.4 M NaCl and 2% β-mercaptoethanol (Sigma)} followed by extraction in 500 µL of chloroform (Sigma- Aldrich): isoamyl alcohol(Sigma- Aldrich) (24: 1). Then 5M NaCl was carefully added and mixed well but gently. The DNA was precipitated in 500 µL 2-propanol (Sigma- Aldrich) and pellet was washed with 70% (v/v) chilled ethanol. The DNA pellet was air dried and resuspended in 100 µL of nuclease free water (NEB) or TE buffer.

Quality of the extracted DNA was analyzed on 0.8% agarose gel (Bio-Rad) stained with ethidium bromide (Invitrogen).

The extracted DNA samples were quantified on Picodrop™ spectrometer at 260 nm. Absorption ratios A_{260}/A_{280}, A_{260}/A_{230} were calculated to characterize the DNA extracts in term of coprecipitation of proteins and organic solvent.

PCR Analysis of Extracted DNA

Usability of the extracted plant DNA was verified by PCR reaction on Bio-Rad thermocycler (T100). Reaction mixtures were prepared as: 50 ng of extracted DNA, 200 µM of each dNTPs (Thermos-scientific, USA), 0.5 µM of each primer, 3 mM of $MgCl_2$ (Thermos-scientific, USA), and 1.0 unit of *Taq* DNA polymerase (Thermos-scientific, USA) along with 1 X *Taq* DNA polymerase buffer (Thermos-scientific, USA), total reaction volume was 25 µL. PCR reaction conditions were optimized as 94 ºC for 5 minutes as initial denaturation followed by 35 cycles, 94 ºC for 1 min denaturation, annealing at 43º C for 1 min and extension at 72 º C for 1 min. Final extension was carried out at 72 ºC for 10 min. The PCR products were visualized on 1.2 % agarose gel.

Restriction Digestion

Restriction digestion of the extracted DNA was performed with 1 U of *Bam*H1 (Thermos-scientific, USA) for 1 hour at 37 ºC in appropriate restriction digestion buffer. The digested DNA was analyzed on 1.0% agarose gel.

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RESULTS AND DISCUSSION

Genomic DNA was successfully isolated by following the modified CTAB method from medicinal plants species of brassicaceae family. The plants had a large amount of secondary metabolites, polyphenols, polysaccharides and proteins that form complex with DNA and are major hindrance in extraction procedure (11, 12). Polyphenolic and polysaccharide contents are much higher in mature leaves as compared to young ones, therefore, in this study mature leaves were used. Five plants out of nine belonged to brassicacaea family and had as high content of essential oils that was a big obstacle in DNA extraction. Different CTAB protocols described in literature (15, 16, 17, and 18) were tried prior for DNA extraction but did not give satisfactory results. The pelleted DNA was very viscous, sticky and could not be resolved and yielded very faint bands on agarose gel electrophoresis (Fig. 1). Brownish color and stickiness indicated that the DNA pellet was coupled with polyphenol oxides and polysaccharides. Other problems encountered to these DNAs were strong effect of contaminations on the downstream reactions such as poor conventional PCR amplification and incomplete enzyme digestion. Polysaccharides, due to their structural resemblance to DNA, contribute functional hindrance to polymerases, ligases and restriction enzymes (13). Polyphenols, when are oxidized by their oxidases, bind with DNA and become difficult to be removed. These contaminants along with oil content make a mesh and trap cellular DNA (10).This might be the main reason for failure of procedures prescribed (15, 16, 17 and 18) for extraction of DNA from brassicaceae plants.

Figure 1: DNA Extractions from *Brassica Nigra* with other Protocols

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Lanes: M: I Kb DNA ladder, 1-3: Dolye and Dolye (1990), 4-6: Khanuja *et al.,* (1999), 7-8: Porebski *et al.,* (1997), 9-11: Dellaporta *et al.,* (1983)

Most of the protocols reported in literature for DNA extraction involve the use of liquid nitrogen and/or complex reagents which are expensive, and need extra phenol/chloroform purifications. Those might work for a particular species but are of no use for Brassicaceae plants. We have cut down all these complications and achieved tremendous results with slight modifications in DNA extraction protocol. Moreover in our findings greater exposure of DNA to complex reagents effects its downstream applications. Fundamentally three steps have been focused in this protocol preparation, 1) Cell wall/membrane removal, 2) Separate DNA from the remaining components of the cell, 3) Maintain the integrity of extracted DNA from nucleases and shearing. Averting liquid nitrogen that greatly effects the cost of overall procedure, cell lysis was performed efficiently with adequate concentration of detergents CTAB and Triton X100 in assistance with physical disruption of the plant tissues (19). Nuclear DNA was protected from the counter effects of nucleases and polyphenols oxides by using higher concentration of antioxidants during grinding as polyphenols and nucleases are released immediately after mechanical stress so direct addition would protect the nuclear DNA. Higher concentrations of CTAB (3%) in combination with high concentration of salt NaCl (6M) overcame the problem of large amount of polysaccharides (14, 20) and enhanced the precipitation of floating DNA. Use of DIECA, combination of PVP and PVPP and β-mercaptoethanol purged the terpenoids, polyketides, phenazine, glycosides and polyphenols (21, 22, 23) efficiently and did not allow those to bind the nucleic acids. Different concentrations of PVP and β-mercaptoethanol were also used with above mentioned protocols but we found no significant effect. Moderate shaking of tubes in between two incubations and with chloroform: isoamyl alcohol facilitated the release of DNA from protein complexes. Complete removal of these proteins is necessary as this generates sticky DNA that interferes downstream processing (21). Due to these measures (mentioned above) after washing (second last step) the DNA pellet obtained was clearly transparent that readily dissolved in water/TE buffer. The DNA shearing effect was found to its minimum that might be due to mechanical grinding of the plant tissues. After the application of said protocol, DNA was extracted efficiently from all plant species. Presence of a single band larger than 10 kb of DNA on 0.8% agarose gel showed high quality of nucleic acids without any noise of RNA and degraded DNA(Fig. 2) that revealed not only good quality of extracted DNA samples but diversity of this protocol.

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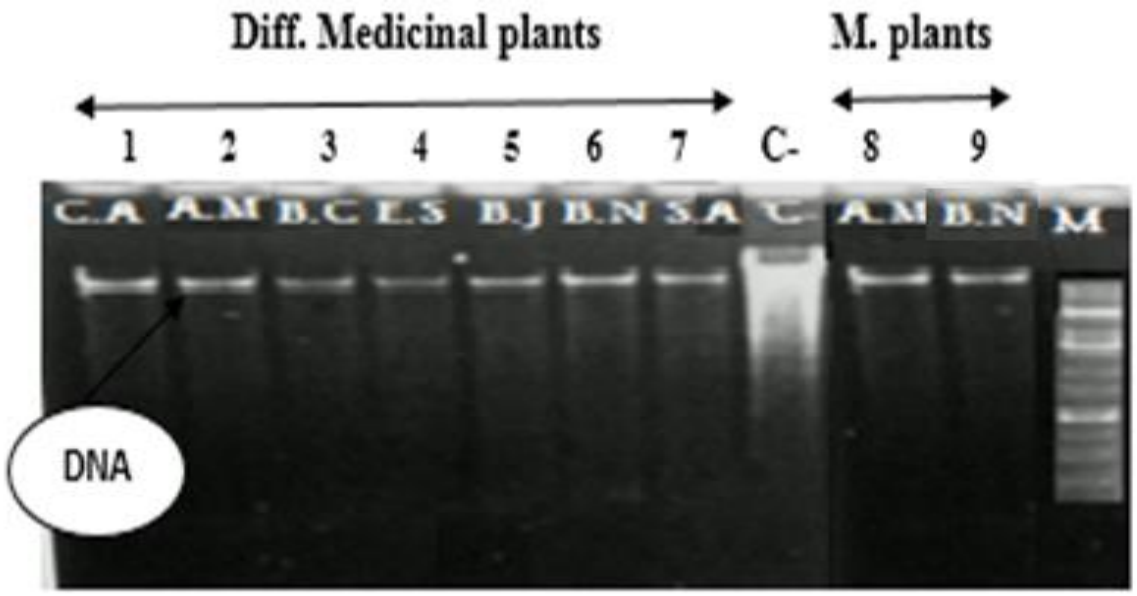

Figure 2: DNA Extracted by Modified CTAB Method from 9 Medicinal Plants

Lanes: M: 1 kb DNA ladder, C.A: *Cirsium arvense*, A.M: *Acacia modesta*, B.C: *Brassica compestris*, E.S: *Eruca sativa*, B.J: *Brassica juncea*, B.N: *Brassica napus*, S.A: *Sonchus arvensis*, A.M: *Alhagi marourum*, B.N: Brassica nigra, C-: DNA without treatment.

Following the successful extraction of DNA from the selected plants purity and concentration of the extracted DNA was determined. Nucleic acids dissolved in nuclease free water were quantified on spectrophotometer by taking absorbance at A_{260}. Ratio of $A_{260/280}$ between 1.7-1.8 indicated the purity of the DNA (Table 2) as these were within the optimal range. A_{260}/A_{230} ratio was also within the range and confirmed that the proteins were efficiently removed. Inexpressive level of other organic compounds omitted the need of phenol chloroform purification steps after extraction. These findings were agreed to the finding of Ihase, *et al.,* (24).

Table 1: T of Extracted DNA from Different Protocols

Protocols	Plant tissue weight (mg)	Purity ($A_{260}/_{280}$)	Quantification (µg/ml)
GF-1 plant DNA extraction kit (Vivantis)	300	1.68-1.7	280-360
CTAB based extraction protocol by Dolye and Dolye (1990)	300	1.4-1.63	99-120
Khanuja *et al.,* (1999)	300	1.4-1.6	78-110
Porebski *et al.,* (1997)	300	1.2-1.3	20-60
Dellaporta *et al.,* (1983)	300	1.53-1.7	89-134

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Table 2: Concentration and A_{260}/A_{280} ratio of extracted DNA

Sr. #	Plant Name	Plant tissue weight (mg)	Plant tissue	A_{260}/A_{280}	Con. µg/ml
1	*Sonchus arvensis*	300	Leaf	1.67	250
2	*Brassica juncea*	300	Leaf, Flower	1.80	359
3	*Eruca sativa*	300	Leaf, Root	1.81	299
4	*Brassica napus*	300	Leaf, Flower	1.78	299
5	*Cirsium arvense*	300	Leaf, Flower	1.74	333
6	*Alhagi maurorum*	300	Leaf, Root	1.86	267
7	*Acacia modesta*	300	Leaf	1.77	380
8	*Brassica campestris*	300	Leaf, Flower	1.73	350
9	*Brassica Nigra*	300	Leaf	1.76	338

The extracted DNA was dissolved in nuclease free water instead of TE buffer as EDTA in TE buffer may confer hindrance to *Taq* DNA Polymerase (25). Successful amplification of the desired genes (Fig. 3) from extracted DNAs verified that the extracted DNA was pure enough to allow DNA polymerase to amplify the targeted region. Positive restriction results with restriction enzymes also confirmed the absence of inhibitory chemicals.

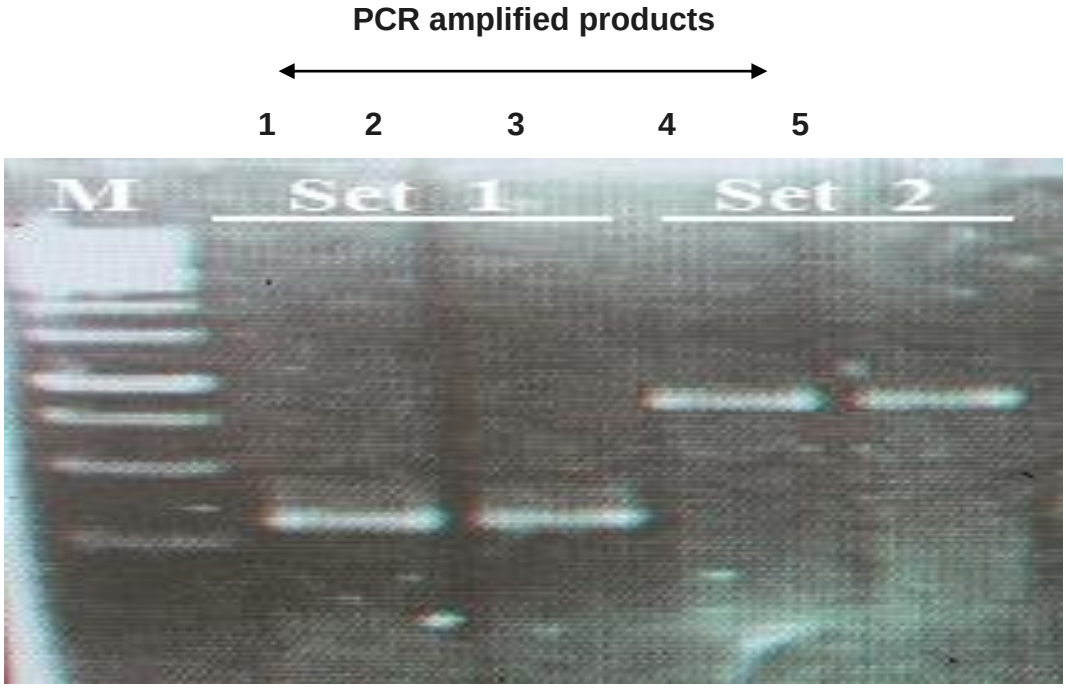

Figure 3: PCR Amplification of Extracted DNA with Two Different Sets

M: 1Kb DNA ladder, 2-3: PCR amplified products from *B. juncea* and *C. Arvense* respectively, 4-5: PCR amplified products from *A. marorum* and *A. modesta* respectively.

In conclusion, we have succeeded to devise a simplest, reliable and cost-effective DNA extraction protocol. The method has efficiently extracted DNA from secondary metabolite rich and chemically heterogenetic medicinal plants in just two hours. Shorter time duration, high throughput both in quality and quantity and use of less toxic reagents has made this method to compete with expensive commercial kits, rather it is better for the

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oily plant species. Moreover, researchers from biological/agricultural sciences can study the plant genomes at low cost by generating less toxic waste.

Suggestions & Troubleshooting

- For dry plants it is better to soak those in distilled water for 8-14 hours at room temperature as rehydration will reverse the process of fabrication that is prominent hindrance in grinding and hence the low yield of extracted DNA. Make sure room temperature must not exceed above 30º C.
- If possible try to avoid the use of petioles and midribs that are rich in polysaccharides and other likewise contaminants. Young leaves can be used as whole.
- In DNA extraction avoid overgrinding as this increases the content of RNA contamination.
- Seeds of these plants are enriched with proteins in addition to polysaccharides and lipids so additional chloroform washing step will markedly increase the yield.
- The process can be delayed after chloroform extraction at any step for upto a week without affecting quality of final outcome.
- Avoid vigorous vortexing as it is main reason of fragmentation of high molecular weight plant DNA. Contents should be mixed by inverting the tubes gently.
- If extracted DNA band is very dim increase the incubation time period in buffer 1 upto 1 hour but not more than that.
- Use the organic solvent (chloroform, isoamyl alcohol, ethanol and 2-propanol) in chilled form should be preferred.

Author Contributions

A. J., F. H., N. A., and M. A. conceived and designed the experiment. A. J. and M. A. analyzed the data. M. A performed the experiments and wrote the paper. All authors read and approved the paper.

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Competing Interests

There is no conflict of interest regarding the manuscript.

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