DNA ISOLATION FROM FRESH AND DRY LEAVES OF SOME MEDICINAL PLANTS

Inderpal KAUR*, Veenu JOSHI*, Shailesh Kumar JADHAV*

* *School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh-492010, India*
Corresponding author: S.K. Jadhav, School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh-492010, India, phone: 0771-2263022, fax:0771-2263022, email: shailesh_07@sify.com

Abstract. DNA extraction is the primary step for carrying out any kind of molecular study. Purity and concentration of DNA are the major factors which affects the success of molecular analysis. In the present study simple and cost efficient method for isolation of high quality DNA was developed utilizing less hazardous chemicals, for fresh and dry leaves of five medicinal plants using five modified protocols to find the best to maximize the purity and concentration of DNA. The modified procedures in *Annona squamosa, Aegle marmelos, Bauhinia variegata, Mimusops elengi, Thevetia peruviana* yielded maximum of 1245, 954, 989, 2019 and 2337 µg/ml from fresh leaves and 128, 610, 126, 257 and 897 µg/ml of DNA from dry leaves respectively without any polysaccharide, polyphenol and RNA impurities.

Keywords: DNA isolation, medicinal plants, dry leaves, cost efficient, purity, hazardous.

INTRODUCTION

Medicinal plants have been used in the treatment of various ailments since prehistoric times and they will remain today and tomorrow the active area of research because of their curative properties towards the ever growing deadly diseases. Such medicinal properties are due to the presence of high amount of polysaccharides, polyphenols and other secondary metabolites. Application of molecular technology can increase and facilitate production of these substances [1] and help save natural resources. Moreover, variations in DNA sequences can be used as character for plant systematic studies and other molecular studies. The success of molecular tools such as molecular markers and genetic engineering techniques are critically dependent on the development of reliable protocol for isolating superior quality DNA.

Although there are several methods available for plant genomic DNA isolation, each plant has some specific requirement for isolation due to variability in their chemical composition i.e. primary, secondary metabolites and pigments which can hinder quality and quantity of isolated DNA. Even closely related plant species belonging to same or related genera may require different isolation protocols [34] for getting high purity DNA. Thus, for each plant group depending upon their secondary metabolite content, an efficient protocol for extraction of high quality DNA is required. Moreover, clarity of results in marker analysis especially RAPD reproducibility depends upon purity of DNA [16].

Foremost step in the isolation of DNA requires disruption of cell wall, cell membrane and nuclear membrane to release all cell contents along with DNA into the extraction buffer which can be done by mechanical grinding followed by treatment with detergents like sodium dodecyl sulphate (SDS) or cetyltrimethylammonium bromide (CTAB). The released DNA is highly susceptible to nucleases enzymes therefore EDTA is used to chelate magnesium ions which are co-factor for these nucleases. Most protein impurities are removed by its denaturation and precipitation using chloroform and/or phenol. RNA on the other hand is normally removed by treatment of the extract with RNaseA enzyme. Polysaccharide-like contaminants can be removed by 0.5 M or more of NaCl [18,20,11].

Generally, DNA isolated from fresh tissues yields good quality and quantity. Whereas in some cases when study demands collection of large number of rare plant samples from distant locations, only dried or stored tissues are available then there is a need to standardize protocol for isolation from the same [15]. In addition, lengthy and costly molecular techniques requires rapid and low cost DNA isolation procedures involving chemicals that are less or not hazardous to the environment and users.

With this point of view the present work aimed at developing DNA isolation protocols by replacing hazardous chemicals and reducing the cost from the available methods for obtaining high molecular weight DNA free of proteins and RNA from fresh and dry leaves of five important medicinal plants.

MATERIALS AND METHODS

Plant material

Five important medicinal plants *Annona squamosa, Aegle marmelos, Bauhinia variegata Mimusops elengi* and *Thevetia peruviana* growing in the campus of Pt. Ravishankar Shukla University, Raipur, Chhattisgarh were selected for the present study (Table1). Fresh and dried leaf samples were collected and thoroughly washed under tap water followed by immersion for 30s in 70% ethanol and blotted dry under folds of sterile filter paper.

DNA isolation protocols

DNA extraction using fresh and dried leaves from five medicinal plants was standardized using five methods, two of which were based on modifications of CTAB method by Doyle and Doyle (1987) [7] and rest three were based on modified method of Prabhu *et al*., (1998) [23].

In Modified CTAB (Doyle and Doyle, 1987) [7] **method 1 –** 1 g of leaf sample was macerated in 4 ml of CTAB extraction buffer comprised of 1M Tris-Cl, 5M NaCl, 0.5 M EDTA, 2.2g CTAB powder and β-Mercaptoethanol and transferred into microfuge tubes and equal amount of chloroform:isoamylalcohol (24:1)

was added. Tubes were then inverted thoroughly to mix the sample till the solution turns milky. Samples were then centrifuged at 12000 rpm for 20 min., supernatant was taken and chilled absolute ethanol twice the volume of supernatant was added and kept at 4^0 C for 20 min. Thereafter, it was centrifuged at 8000 rpm for 10 min. and supernatant was discarded and pellet was collected. Pellet was then washed with 70% ethanol, dried and dissolved in 1X TE buffer.

In Modified CTAB (Doyle and Doyle, 1987) [7] **method 2 –** all the steps were same as previous method except incubation at 65^0 C for 20 min. after maceration in buffer and addition of 200 µl of 3M sodium acetate and 600 µl of chilled isopropanol after C:I treatment for precipitation instead of absolute ethanol.

In Modified methods of Prabhu *et al.*, (1998) [23] **–** 1g of leaves crushed in 4 ml of pre heated extraction buffer (1M Tris-Cl, 5M NaCl, 0.5 M EDTA) containing different detergents in three different modified methods (**1**. Sodium sulphite **2**. Sodium carbonate and **3**. Di sodium hydrogen phosphate) instead of CTAB, then were transferred to microfuge tube and incubated at 65^0 C for 10 min. followed by vortexing for 3-4 times and immediately put on the ice for 2 min. Thereafter, these were centrifuged at 12000 rpm for 12 min. and supernatant was collected to which double amount of chilled isopropanol was added and again centrifuged at 12000 rpm for 10 min. The pellet obtained was dissolved in the 100 µl of 3M sodium acetate and 600 µl of chilled absolute ethanol and again centrifuged for 8 min. at 8000 rpm. Finally the pellet obtained was washed with 70% ethanol, dried and dissolved in 1X TE buffer.

Estimation of quantity and purity of DNA

The yield of DNA per gram of leaf tissue extracted was measured using a UV-VIS Spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA samples were then electrophoresed on a 0.8% agarose gel, visualized and photographed by Gel Documentation system.

Calculation of cost of isolation per sample

Cost of DNA extraction per sample was calculated by summing up the cost of each constituent of the extraction buffer used in the isolation of 1g plant leaf sample.

RESULTS

Out of the different modified methods used for isolation of DNA in *Annona squamosa* maximum purity (1.79, 1.59) along with high concentration (1245, 128 µg/ml) was observed with modified method using sodium carbonate in fresh as well as dry leaves respectively. The same method has been found good in *Aegle marmelos* with best purity (1.82, 1.64) and concentration (954, 610 µg/ml) for fresh and dry leaves respectively. In *Bauhinia variegata* best DNA purity (1.82, 1.70) and concentration (989, 126 µg/ml) has been observed in modified method using sodium sulfite in fresh and dried leaves respectively. Fresh leaves of *Mimusops elengi* gave best purity (1.82) and maximum concentration (2019 µg/ml) with modified CTAB method 2 while dried leaves gave maximum purity (1.68) and concentration of 257µg/ml with modified method using sodium sulfite. In *Thevetia peruviana* again best purity 1.90 and maximum concentration 2337 µg/ml was observed with modified method using sodium sulphite which is similar to results found in sugarcane [1] and citrus leaves [3]. Dried leaves of *T. peruviana* gave maximum purity (1.76) and concentration (897µg/ml) with modified method using di- sodium hydrogen phosphate (Table 2).

Among fresh and dried leaves, fresh leaves always gave high purity and concentration in all the plants but present protocols proved quite successful in providing good purity and concentration in dried leaves also.

Moreover, cost of extraction per sample (IR 8.16, 8.17/-) in modified CTAB (Doyle and Doyle, 1987) method 1 [7] and method 2 respectively was found to be same which is much higher than that involved in modified methods using sodium sulphite (IR 0.80/-), sodium carbonate (IR 0.83/-) and di- sodium hydrogen phosphate (IR 0.81/-) (Table 3). Also, modified CTAB method 1 (55min) and modified CTAB method 2 (75min) were found to be much more time consuming in comparison to rest three methods (45min).

In addition, all the five extraction protocols did not differed much in the number of steps involved. About use of toxic chemicals, modified Prabhu *et al.*, (1998) methods [23] did not involve any toxic and hazardous chemicals whereas in modified CTAB method chemicals like phenol, B-merceptoethanol were used (Table 2).

Table1. Details of medicinal plants under study

Scientific Name	Common Name	Family	Medicinal Properties	Secondary Metabolite Content
Annona squamosa	Sugar apple, sitaphal	Magnoliacea	Antigenotoxic [31], anti head lice [32], anti thyroidic [19], antifertility [17]	Glycoside, alkaloids, saponins, flavonoids, tannins, carbohydrates(polysaccharides), proteins, phenolic phytosterols, amino acids [12]
Aegle marmelos	Bel	Rutaceae	Antioxidant [29], antiulcer [28], antidiarrohea [13], antidiabetic [2]	Alkaloids, cardiac glycosides, terpenoids, saponins, tannins, flavonoids and steroids[28]
Bauhinia variegata	Kachnaar	Fabaceae	Antidiabetic [33], hepatoprotective [5], anti-tumour [25]	Saponins, tannins, flavonoids, glycoside, phenolics, phytosterols [35]
Mimusops elengi	Spanish cherry, Maulshri	Sapotaceae	Anti-anxiety and anticonvulsant [8], antipyretic [24], Anti-atherosclerotic [27]	alkaloids, tannins, ursolic acid, steroids, querrcitol, lupeol and mixtures of triterpenoid saponins [24]
Thevetia peruviana	Yellow kaner	Apocynaceae	Antimicrobial [21], antifungal [9], antitermite [14], antispermatogenic [10]	Flavonoids, glycosides, phlobatannins, saponins, steroids and tannins [21]

Table 2: Quantitative estimation of DNA concentration & purity in medicinal plants using different extraction methods

2.1. ***Annona squamosa***

Extraction protocols	Purity		Concentration µg/ml	
	Fresh leaves	Dry leaves	Fresh leaves	Dry leaves
Modified CTAB method 1	1.62	1.52	840	128
Modified CTAB method 2	1.67	1.54	842.5	100
Modified method using sodium sulfite	1.65	1.5	1315.5	110
Modified method using sodium carbonate	**1.79**	**1.59**	**1245**	**128**
Modified method using di- sodium hydrogen phosphate	1.75	1.53	180	150

2.2. ***Aegle marmelos***

Extraction protocols	Purity		Concentration µg/ml	
	Fresh leaves	Dry leaves	Fresh leaves	Dry leaves
Modified CTAB method 1	1.75	1.56	867	198
Modified CTAB method 2	1.72	1.57	976	562
Modified method using sodium sulfite	1.78	1.56	1327.5	252
Modified method using sodium carbonate	**1.82**	**1.64**	**954**	**610**
Modified method using di- sodium hydrogen phosphate	1.87	1.65	1054	395

2.3. ***Bauhinia variegata***

Extraction protocols	Purity		Concentration µg/ml	
	Fresh leaves	Dry leaves	Fresh leaves	Dry leaves
Modified CTAB method 1	1.68	1.54	193	145
Modified CTAB method 2	1.75	1.55	198	252
Modified method using sodium sulfite	**1.82**	1.54	**989**	123
Modified method using sodium carbonate	1.84	**1.70**	210	**126**
Modified method using di- sodium hydrogen phosphate	1.84	1.60	345	112

2.4. ***Mimusops elengi***

Extraction protocols	Purity		Concentration µg/ml	
	Fresh leaves	Dry leaves	Fresh leaves	Dry leaves
Modified CTAB method 1	1.77	1.67	871	201
Modified CTAB method 2	**1.82**	1.65	**2019**	120
Modified method using sodium sulfite	1.89	**1.68**	1210	**257**
Modified method using sodium carbonate	1.90	1.62	1250	765
Modified method using di- sodium hydrogen phosphate	1.91	1.56	1256	193

2.5. ***Thevetia peruviana***

Extraction protocols	Purity		Concentration µg/ml	
	Fresh leaves	**Dry leaves**	**Fresh leaves**	**Dry leaves**
Modified CTAB method 1	1.82	1.72	897	245
Modified CTAB method 2	1.89	1.74	2082	562
Modified method using sodium sulfite	**1.90**	1.75	**2337**	501
Modified method using sodium carbonate	1.92	1.75	2010	876
Modified method using di- sodium hydrogen phosphate	2.00	**1.76**	2117	**897**

Table 3. Cost, number of steps and removal of toxic chemicals involved in different protocols

Extraction protocols	Cost per sample (IR)	No. of steps involved	Time duration (minutes)	Removal of hazardous compound
Modified CTAB method 1	8.16/-	7	55 min	Phenol
Modified CTAB method 2	8.17/-	7	75 min	Phenol
Modified method using sodium sulfite	0.80/-	8	45min	β- Merceptoethonal, Phenol, Chloroform, Isoamylalcohol
Modified method using sodium carbonate	0.83/-	8	45min	β- Merceptoethonal, Phenol, Chloroform, Isoamylalcohol
Modified method using di-sodium hydrogen phosphate	0.81/-	8	45 min	β- Merceptoethonal, Phenol, Chloroform, Isoamylalcohol

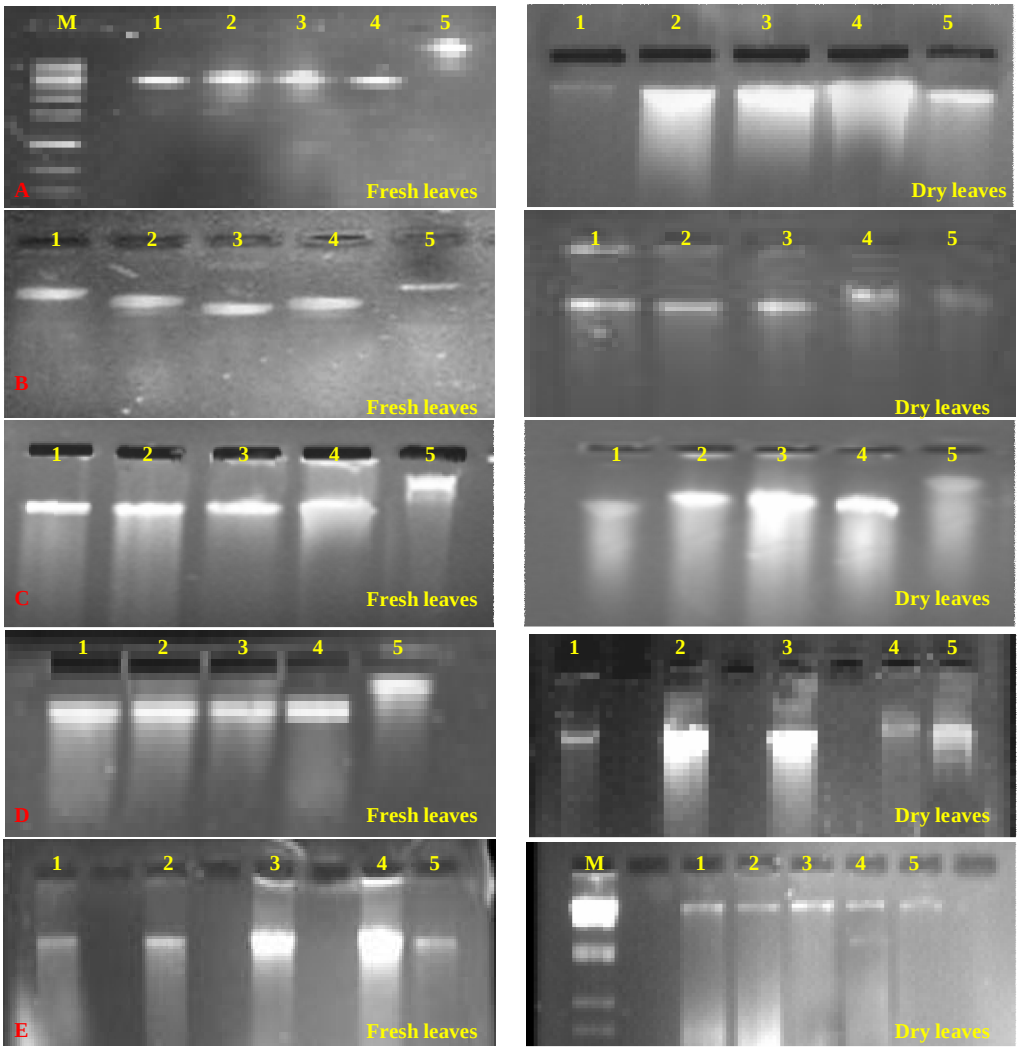

Figure 1: Gel run of genomic DNA
(Isolated by **A.** Modified CTAB method 1, **B.** Modified CTAB method 2, **C.** Modified method using sodium sulphite, **D.** Modified method using sodium carbonate, **E.** Modified method using di- sodium hydrogen phosphate for fresh and dry leaves of 1-*B. variegata*, 2-*M. elengi*, 3-*T. Peruviana*, 4-*A. marmelos*, 5-*A. squamosa*).

DISCUSSIONS

Isolation of high molecular weight DNA is the primary requirement for applying any kind of molecular biology techniques in the study and complete utilization of medicinal plants. Modified methods of CTAB- Doyle and Doyle (1987) [7] and methods using sodium sulphite, sodium carbonate and di-sodium hydrogen phosphate were used on five selected medicinal plants *Annona squamosa, Aegle marmelos, Bauhinia variegata, Mimusops elengi, Thevetia peruviana.* Modified method using sodium carbonate was found to beneficial in fresh as well as dry leaves of *Annona squamosa* and *Aegle marmelos* due to its strong buffering and detergent properties. In *A. marmelos* this method produced far greater concentration of DNA than the previously reported yield of 42 µg/ml using CTAB [4]. In fresh and dried leaves of *Bauhinia variegata,* dry leaves of *Mimusops elengi* and fresh leaves of *T. peruviana* modified method using sodium sulfite was found to be beneficial, which may be due to reducing action of sodium sulfite for polyphenol oxidase which prevents the production of polyphenolic compounds. Similarly, the incorporation of sodium sulfite in extraction buffer proved beneficial in increasing the yield and purity of DNA in *Acacia* species [6], *Allium stracheyi* [26], Sugarcane [1] and *Citrus* leaves [3]. Fresh leaves of *Mimusops elengi* gave best results with modified CTAB method 2 while dried leaves of *T. peruviana* produced best results with modified method using di-sodium hydrogen phosphate as it is an anionic salt with profound buffering action which regulates the pH (Table 2). In all the five plants fresh leaves always gave more purity and concentration irrespective of the method used which is in accordance to reports by earlier researchers [22]. Therefore, in comparison to CTAB method of Doyle and Doyle (1987) [7], methods of Prabhu *et al*. (1998) [23] proved more effective in producing high quality and concentration of DNA in fresh as well as dry leaves. Moreover, the cost of DNA isolation was also found to be less in methods Prabhu *et al*., (1998) [23]. Out of all five plants *Thevetia peruviana* gave good quality DNA [26], Sugarcane [1] and *Citrus* leaves [3]. Fresh leaves of *Mimusops elengi* gave best results with modified CTAB method 2 while

dried leaves of *T. peruviana* produced best results with modified method using di- sodium hydrogen phosphate as it is an anionic salt with profound buffering action which regulates the pH (Table 2). In all the five plants fresh leaves always gave more purity and concentration irrespective of the method used which is in accordance to reports by earlier researchers [22]. Therefore, in comparison to CTAB method of Doyle and Doyle (1987) [7], methods of Prabhu *et al*. (1998) [23] proved more effective in producing high quality and concentration of DNA in fresh as well as dry leaves. Moreover, the cost of DNA isolation was also found to be less in methods Prabhu *et al*., (1998) [23]. This is due to high polysaccharide content of *A. squamosa* leaves along with other secondary metabolites which has also been reported by other investigators [12, 15]. Therefore, the present study standardizes DNA isolation protocols for five important medicinal plants using fresh and dry leaves. Out of the five different protocols modified Prabhu *et al*., 1998 [22] methods were found to be effective in producing pure DNA in all the plants except *M. elengi.* Moreover, these methods were also found to be cost effective as well as less time consuming in comparison to modified CTAB methods.

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