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Rapid and Simplified Method for Isolation of DNA from Fresh Leaves, Herbarium, Silica Dried and Tubers Sample of Medicinally Important Plant Species Found in Indian Forest.

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***Abstract*--A simple and efficient protocol for isolating genomic DNA from fresh leaves, Herbarium and silica gel dry leaves of 41 medicinal plant of was developed. It involves a modified CTAB procedure using 15 % CTAB, 10 % -mercaptoethanol, 1.5 M NaCl and 7% PVP. The extraction was carried out at 65°C. A slight increase in the concentrations of these chemical components helped in the removal of secondary metabolites and polysaccharides from the DNA preparation when we used tubers also. The quantity and purity of isolated DNA was higher when compared with DNA extracted by the methods of Doyle and Doyle (1990). The DNA yield ranged from 357-12.1 µg per g of depending upon sample used for isolation. 10 medicinal plants need further modification in protocol. The DNA samples were found suitable for analysis with nuclear ITS Amplification.**

Key word- Medicinal plant, Secondary metabolites, nuclear ITS, amplification

I. INTRODUCTION

Molecular techniques have been employed to authenticate medicinal materials since the middle of 1990s. Molecular techniques, such as DNA fingerprinting, DNA sequencing and DNA microarray, have been applied extensively to authenticate medicinal materials with a number of these applications having been patented and commercialized.DNA sequencing can retrieve the maximum molecular information from a particular DNA region. Polymorphism of nucleotide sequences provides information to distinguish closely related species from distantly related species and between genuine medicinal materials and adulterants. All this molecular marker techniques are divided into three parts. First part, isolate DNA from a wide range of biological samples including processed foods (e.g., medicinal plant, material from forest, local market, canned, partially cooked, pickled, salted or smoked). Second, a specific segment of DNA is amplified using PCR. Third, the nucleotide sequence of the amplified segment of DNA is determined. But large number of medicinal plant species produces secondary metabolites such as alkaloids, flavonoids, phenol, gummy polysaccharides, terpenes, quinines and saponines etc. These secondary metabolites contaminated and decreased the quality of DNA during isolation and its subsequent amplification. Isolation of genomic DNA from plant producing high amount of secondary metabolites is a tedious task, requires gradient centrifugation system and hazardous solvent like carbon tetrachloride and n-hexane.. The problems encountered in the isolation and purification of genomic DNA from medicinal and aromatic plant, could be now minimized as this protocol permits isolation of genomic DNA from leaves, tubers from medicinal plant species, giving fairly good yield of genomic DNA. The isolated DNA was able to provide good PCR amplification product for further sequencing process and other marker analysis.(Michiels et al., 2003, Khanuja et al., 1999, Tel-Zur et al., 1999).

II. MATERIAL METHOD

We have collected 41 different medicinal plant species and selected different types of sample for study like fresh tubers, leaf, silica dried specimens and herbarium for DNA isolation from medicinal plant commonly found in Indian forest. Out of which 10 plants need additional modification in protocol while remaining 31 plants gave result without modification. We marked these plant with (*) in table -1. As in Ayurved various parts are used as medicine like leaves, tubers we used different part for our study. We recommended that these plants (*) kept two days in dark.Middle rib was removed from fresh leaf. Tubers were properly cleaned and upper layer was removed. DNA was extracted by using following steps.

- Wash the tissue with autoclave water and wiping it with 70 % alcohol and dry
- Grind 500 mg fresh leaves or 30 mg silica dried or 30 mg herbarium and 400 mg of tuber sample for DNA isolation
- Grind plant tissue into powder with liquid nitrogen.
- Add 700 µl of pre warm DNA extraction buffer (CTAB (15 %), EDTA (50mM), NaCl (1.5M), TrisCl (100 mM), RNase (40µg/ ml), β – Mercaptoethanol (10 %)), (RNAse 70 µg/ ml,optional). vertex for 1 min. and incubate at 65^{o} C for 30 to 40 min with gentle mixing after every 10 min.
- Centrifuge at 8000 rpm for 5 min. if marked (*) plants some time not properly centrifuged then add 30 µl of 5 M Hepes or 30 µl of 5% Bovine serum albumin and 20 µl of 7 % PVP. Stand for 5 min and centrifuge.
- Transfer the supernatant to fresh 1.5 ml centrifuge tube, (*) Add 20 µl of 8 N NaCl. Add equal quantity

of Chloroform: Isoamyl alcohol (24:1). Repeat the step if necessary.

- Centrifuge at 8000 rpm for 5 min.
- Carefully transfer the upper aqueous layer into another 2 ml centrifuge tubes.
- Add equal quantity of ice-cold ethyl alcohol and centrifuge at 10000 rpm for 5 min.
- Discard the supernatant and air dry the pellet
- Wash the pellet with 70% alcohol (two times if necessary)
- Air dry and then dissolve the pellet in 50 µl of TE buffer.
- Result absorbed on 0.8 % agarose gel at 100 for 30 t0 40 min.(Figure1a and Figure 1b)
- The yield and quality of DNA per gram of tissue calculated by taking ratio 260/280 nm. (Table 1)

For Amplification of ITS-2 sequence

Two universal primers, *ITS2F:* 5'-ATGCGATACTTGGTGTGAAT-3and ITS3R: 5'-GACGCTTCTCCAGACTACAAT-3' were used for PCR. PCR was carried out in 25 µl volume reaction mixture. The reaction mixture contained 25 ng of DNA, 1 U Taq DNA polymerase enzyme (Fermentas, USA), 100 mMdNTP mix, 1.5 mM $MgCl_2$, 1X Taq DNA polymerase buffer and 10 mM Primer, the `plant DNA was amplified using the following conditions with initial denaturation of 94^{o}C for 5 min, followed by 30 cycles , denaturation at 94^{o}C for 30 s, annealing at 55^{o}C for 30s, extension at 72 ^{o}C for 45s, and final extension 72^{o}Cfor 5 min. The amplification is checked by loading the sample on 1% agarose gel.

III. Results and Discussion

Firstly, the success of molecular markers identification is highly dependent on the quality and amount of the reference DNA. To isolate pure and intact DNA from plant tissues,numerous protocols have been established (Saghai-Maroof et al. 1984; Doyle and Doyle 1990; Scott and Playford 1996; Sharma et al. 2000; Pirttilä et al. 2001; Drábková et al. 2002; Shepherd et al. 2002; Mogg and Bond 2003; Haymes 1996).The isolation of genomic DNA from plant species is faced with problems that include: 1) degradation of DNA due to endonucleases, 2) co-isolation of highly viscous polysaccharides, and 3) co-isolation of inhibitory compounds has polyphenols and other secondary metabolites, which directly or indirectly interfere with enzymatic reactions.(Barzegari et al., 2010, Tivari et al., 2008, Vinay et al., 2005). During standardization of this protocol we considered the two things first one is amount of DNA and quality**,** however, plant species belonging to the same or related genera can exhibit enormous variability in the complexity of pathways of dispensable functions. Thus, these DNA extraction protocols cannot be reproduced for all plant species; we overcome this problem to same extend by slight increasing concentration of chemicalcomponents of extraction buffer, we are used total 41 plant species for DNA Isolation, which are belongs to different Families and Genera (Table1 and Table 2), out of these 41 species31 species gives result with our protocol (Table 1), but for 10 plant species we suggested modification (Table 2)(Porebski et al. 1997; Ribeiro and Lovato 2007). Highest 370 µg/ml and lowest 55µg/ml DNA yield from Fresh leaves, while 123.8 µg/ml highest and 22.8 µg/ml lowest yield of DNA from tubers and 55µg/ml highest and 19.2 lowest yield of DNA from Herbarium sample, from silica gel preserved sample highest 62.1 µg/ml and lowest 28.5 µg/mlyield of DNA were got with normal protocol, the quantity of DNA from herbarium and silica gel preserved sample is depends on condition of herbarium as preserved sample, and it is increased if herbarium were in good condition (table 1). Sample required modification gives low quantity of DNA µg/ml, Fresh leaves gives highest of 97.6µg/ml and lowest of 23.4µg/ml of DNA yield, from tuber 50-45 µg/ml while 20-21.6µg/ml from herbarium sample and 36-20 µg/ml from silica gel preserved sample (Table 2). Generally in each step 80 % of DNA is recovered, so when minimize the number of step we got a good quantity of DNA per Gram weight of sample; Simultaneously we have reduced the time required for isolation of DNA, which is helpful for processing of large number of sample within shorter time period. The method offered in this paper eliminates much of the laborious and time-consuming steps of most other protocols. Simultaneously suggestion given before starting protocol also helpful for increased yield of DNA. We used of hepes or bovine serum albumin which remove polyphenols from plant. We report an improved technique of DNA extraction from plants with high levels of polyphenols and polysaccharides. (Virupakshi and Naik, 2007 and Xiang et al., 2002)The DNA resulting from the optimized protocol was observed to be largely free from polyphenolics and secondary metabolites, as determined by successful digestion with restriction endonucleases and PCR amplification. All methods have in common the use of detergents such as CTAB for cell wall lysis, but for optimum cell lysis we used higher percentage of CTAB, up 15 % w/v. However, RNA-free DNA can be obtained by treating the extracts with RNAse A. The presence of RNA in DNA extracts is not a major problem as this usually does not interfere with PCR or restriction digestion, and it is removed from DNA preparation by using RNAsetreatment (70 µg/ml). In conclusion, the isolated DNA yielded high quantities from small amounts of plant tissue, for study of molecular markers. The working protocol presented proved to be valid and suitable for a wider range of medicinal plant research and should also be applicable in high throughput for routine DNA studies in minimum equipped laboratories.

IV. Abbreviations

CTAB: Cetyltrimethylammonium bromide; DNA: Deoxyribonucleic acid; EDTA: Ethylenediaminetetraacetic acid; gDNA: Genomic DNA; PCR: Polymerase chain reaction; Tris: Tris(hydroxymethyl)aminomethane; RNA: Ribonucleic acid; Mg:milligram, PVP: polyvinaylpyrrolidone; F.L: fresh leaves; H.S.: Herbarium sample; S.G.L:Silica Gel Dried; Concen.:concentration.

V. AUTHORS CONTRIBUTION

KK performed and assessed the DNA extraction methods, and helped to draft the manuscript. PT provided technical advice and provided critical revisions of the manuscript. HK provide medicinal plant material and provided technical advice.

VI. ACKNOWLEDGEMENTS

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A. Endnote

15% CTAB, 1.5MNaCl, 50mM EDTA, 100 mmTris–HCl, 1% polyvinylpyrrolidone, 10 % β-mercaptoethanol, 40 µl RNase

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Table 1- List of medicinal plant selected for DNA isolation, (F.L- fresh leaves, H.S. –Herbarium sample, S.G.L.- Silica Gel Dried sample, Concen.-concentration).

Sr. No	Name of medicinal plant	F.L.	Tuber	H.S.	S.G.L.
1	*C. borivillianum*	301.2	102	26.4	35.2
2	*C. laxusm*	324.6	97.5	33.4	44.1
3	*C. aurenudacium*	336.7	120.8	54.3	62.1
4	*C. tuberosum*	370.5	79.9	32	52
5	*D. petaphyla*	200.1	-	-	-
6	*D. Atunata*	230.4	-	-	-
7	*A. racimosa*	210.4	67.3	-	-
8	*G. superba*	98.2	-	-	-
9	*H. rosa-sinensis*	180.4	-	-	-
10	*P. zeylanica*	80.9	-	-	-
11	*P. Indica*	60.02	-	-	76
12	*A. vasica*	86.6	-	-	-
13	*C. malbaricum*	344.9	120.8	22.3	57.3
14	*C. attanetum*	357.5	167.4	40.1	60.2
15	*C. glacum*	330.8	87.6	36	35.3
16	*C. nimmonii*	349.6	95.8	52.1	32.9
17	*C. glycoidus*	303.6	101.2	23.1	32.6
18	*C. gotanase*	308.9	123.8	29.3	38.7
19	*C. neplanse*	301.4	120.5	28	32.7
20	*C. comosum*	289.6	135	21.5	29.6
21	*C. belgaumense*	89.6	65.9	22.2	28.5
22	*C. breviscapum*	288.7	86.7	24.2	33.4
23	*C. hynum*	198.7	-	19.2	32.9
24	*C. decipiens*	78.9	23.1	19.3	-
25	*C. longa*	67.4	22.8	31.5	-
26	*C. zedoaria*	55.6	19.1	21	-
27	*C. papaya*	200.6	-	35.1	43.8
28	*C. alata*	120.3	-	-	-
29	*A. precatorius*	122	-	-	-
30	*T. cardiofolia*	95.1	-	-	-
31	*E. officinalis*	87.3	-	-	-

Table 2- List of medicinal plant selected for DNA isolation. Marked plants (*) need modification during DNA isolation (F.L- Fresh leaves, H.S. – Herbarium sample, S.G.L.- Silica Gel Dried sample, Concen.-concentration).

Sr. No	Name of medicinal plant	F.L.	Tuber	H.S.	S.G.L.
1	*T. arjuna**	50.8	-	-	-
2	*C. bharuchii **	97. 6	45.3	20.1	36.2
3	*C. kolhapurens **	88.4	50.1	21.3	32.6
4	*C. procera **	30.77	-	-	-
5	*F. racemosa **	23.4	-	-	-
6	*A. hetrophylous **	58.2	-	-	-
7	*F. benghalensis**	49.5	-	-	-
8	*A. americana **	43.3	-	-	20.1
9	*S. indiaca **	67.4	-	-	-
10	*D. quercifolia **	62.1	-	-	26.6

Figure-1a: Isolation of DNA from fresh leaves, in lane 1 λ- ecoRI and HinDIII digest, from lane 2 to lane 16, C. borivillianum, C. laxusm, C. aurenudacium, C. decipiens, C. tuberosum, D. petaphyla, D. Atunata, A. racimosa, C. papaya, ,C. longa, C. zedoaria, C. alata, H. rosa-sinensis, D. Atunata, A. precatorius.

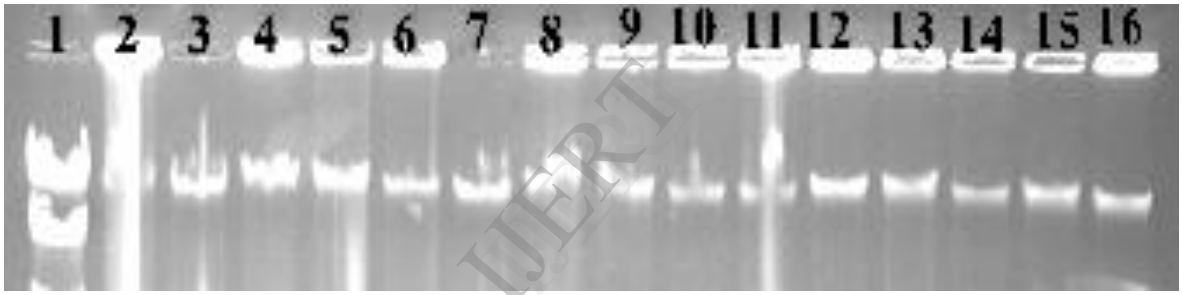

Figure-1b: lane 1 λ- ecoRI and HinDIII digest, from lane 2 to lane 11 fresh leaves sample,C. bharuchii, C. kolhapurens, C. procera,F. racemosa,A. hetrophylous, F. benghalensis, A. americana, S. indiaca, D. quercifolia, T. arjuna, from lane 12 to lane 16 silica gel preserved sample of C. bharuchii, C. kolhapurens, A. Americana,D. quercifolia.