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Efficient DNA extraction protocol suitable for molecular authentication of medicinal herbs

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Abstract
Medicinal plants are being used since from the days of yore for the treatment and management of human, animals and plant diseases. Although there was a slump in their use for some period, it is re-attaining popularity due to its unmatched benefits. The enormous demand at both national and international market has resulted in necessity to maintain the purity and authenticity of plants. As most of the herbal medicines are administered in dried form, identification and authentication is becoming difficult. The current molecular methods to identify such material demand quality DNA for downstream molecular analysis. Obtaining the quality DNA from the available extraction protocols in selected medicinal herbs is troublesome due to the interference of plant secondary metabolites. There are quite a few publications in which specific protocols for isolation has been defined for a particular family or species of medicinal herbs. The prior art describes various protocols for DNA extractions in fresh and dried samples, whereas the extension of the same methods universally to obtain quality DNA from dry samples was either unsuccessful or time-consuming and expensive. This necessitates us to develop and optimize the efficient and economical quality DNA extraction protocol. The resulted modified protocol is rapid, simple and effective for dried plant material and very useful for further molecular methods for authentication. This method does not require expensive chemicals such as proteinase K, liquid nitrogen etc. The extracted DNA was also suitable for PCR and RAPD, ISSR proofing.

Keywords: Medicinal plants, Molecular authentication, DNA isolation, PCR amplification, ISSR analysis

Introduction
Botanicals have been globally used since from the ages to treat and manage the medical infirmity. Over the period, their usage registered a decline perhaps due to emergence of synthetic antibiotics and drugs [1]. However, the harmful effects associated with synthetic drugs and antibiotics have brought the herbal medicine again to the forefront. According to WHO survey reports, round 80 per cent of the global population still relies on plants for their medical conditions. The growing preferences in herbal medicines in both developed and developing countries recorded impact on the global economy. An herbal medicinal market is considered to constitute the mostly rapidly growing segment generating billions of dollars in trade [2].

The botanical medicines are extensively used in Ayurveda, Unani and Homoeopathic medicine. The therapeutic plants *Eclipta alba* (Asteraceae), *Azadirachta indica* (Meliaceae), *Leucas aspera* (Lamiaceae), *Terminalia arjuna* (Combretaceae), *Phyllanthus amarus* (Euphorbiaceae), *Tinospora cordifolia* (Menispermaceae), *Aegle marmelos* (Rutaceae), *Curcuma longa* (Zingiberaceae), *Withania somnifera* (Solanaceae), *Piper nigrum* (*Piperaceae)* are the most widely and extensively used in all kinds of medical practices.

About 50 per cent of all the drugs in clinical use in the world are derived from phytochemicals and their analogues [2]. The Encyclopaedia of Chinese materia medica published in 1977, lists nearly 6000 drugs out of which 4800 are of plant origin. According to FAO reports, the modern pharmacopoeia still contains at least 25 per cent drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants. The medicinal herbs produce simple to complex chemical compounds which acts individually or in combinations to synergistically enhance the therapeutic action [3]. This includes anti-inflammatory compounds, phenolic compounds act as antioxidants, tannins act as antibacterial substances, alkaloids act as stimulants, antitumor activity. Flavonoids acts as anti-allergic, anti-inflammatory, antidiarrheal activities, Antipyretic, Expectorant [4, 5]. Many pharmacological properties are evidenced from the history such as antiviral, antibacterial, antioxidant, anti myotoxic antiheamorrhagic and known to be used as rejuvenating tonic [1].

However, the adulteration and use of spurious materials as substitutes have become a major concern for users and industry for reasons of safety and efficacy. The credibility of any system of medicine depends on the quality of medicine administered. The correct identification and authentication of plants forming the drug is a prerequisite and fundamental to whole realm of medicine and science. Therefore, authentication of medicinal plants is of utmost importance to maintain efficacy and therapeutic property of the preparations in which these plants are used. There are some efforts have been made to identify adulterants medicinal plants and to ensure its purity and quality [6].

The current molecular methods used to identify and authenticate such material demands high quality of DNA, which is almost difficult to obtain from dried plant material. The secondary metabolites, polyphenols, RNA and proteins tend to interfere in the isolation of DNA from the dry powder [7]. These secondary metabolites were also interfering in precipitation reactions which reduces the DNA yield and leads to poor PCR amplification (Porebski *et al.*, 1997). The extraction of genomic DNA from succulent plants (Diadema *et al.* 2003) [10] and dried plant material is time consuming and difficult, which results in poor DNA purity (Khan *et al.*, 2007). The poor DNA quality is not feasible for downstream molecular manipulations (Varma *et al*., 2007 and Ahmad *et al*., 2004) [11, 21,].

There are many extraction protocols published and documented to isolate DNA from fresh and dried plant tissue containing high amount of secondary metabolites, polyphenols, RNA and proteins. Choudhary *et al.*, 2008 [12], noticed a quality DNA extraction from *Vigna* sp. suitable for RAPD and restriction digestion by adopting a slight modification in concentration of reagents used in general DNA extraction method. Further, the use of expensive chemical like CTAB for DNA purification results in high quantities of pure DNA from a fresh plant samples containing large amounts of secondary metabolites and essential oils (Suman *et al*., 1999) and from latex-containing plants. The widely suitable DNA extraction process can't be followed and is well understood from the literature, most of the plants require relevant and specific protocols for DNA extraction. Doyle and Doyle (1990) used CTAB for DNA extraction along with ß-mercaptoethanol and Proteinase K. Stein *et al*. (2001) [17] have used RNase per 200-300 mg of the sample and CTAB and SDS were used in combination by Keb-Llanes *et al*. (2002) [18]. The glucose as reducing agent along with CTAB was used in the protocol identified by Permingeat *et al*. (1998). Various extraction protocols are available for isolation of DNA, however many of them require a large amount of fresh material, expensive reagents and is time consuming.

Krizman *et al*. (2006) [20] used activated charcoal, sorbitol and liquid nitrogen in extraction buffer for DNA isolation from herbarium specimens, which include mechanical grinding of the sample as a critical step, the method found suitable for the isolation of good quality DNA from various herbarium specimens for polymerase chain reaction (PCR) based downstream applications. However, the method was found to be not economical, as it involves expensive chemicals and time-consuming process. Although such several successful DNA extraction protocols for plant species containing polyphenolics and polysaccharides compound have been developed, none of these are universally applicable to all plants (Varma *et al.*, 2007) [11, 21]. The current paper details about the successful effort made in development of new efficient and economical protocol for DNA extraction from various dried medicinal plants species. The identified protocol is effective in isolating the quality DNA from different plant species with excellent yields, and the isolated DNA is suitable for PCR and ISSR analysis.

Materials and method

Plant material

The commercially available dry samples of different brands in packed forms were obtained from local markets in Mysore and are listed in the table.1

Table 1: DNA extraction status and PCR amplification of market samples**.**

S. No.	Sample	DNA Extraction			PCR amplification
		+/-	µg/mg	OD 260/280	
1	*A. indica*	+	800	1.79	+
2	*A. marmelos*	+	758	1.78	+
3	*E. alba*	+	1080	1.8	+
4	*L. aspera*	+	921	1.82	+
5	*P. nigrum*	+	712	1.79	+
6	*P.amarus*	+	752	1.8	+
7	*T. arjuna*	+	812	1.9	+
8	*T. cordifolia*	+	835	1.8	+
9	*C. longa*	+	756	1.81	+
10	*W. somnifera*	+	865	1.78	+

Chemicals and reagent

The chemicals like Tris-HCl, Ethylenediaminetetraacetic acid, Sucrose, Triton X and β-Mercaptoethanol, PCR grade dNTPs, enzyme *Taq* DNA Polymerase, 10X-assay buffer for *Taq* DNA Polymerase, primers, Magnesium chloride and agarose used for gel electrophoresis were obtained from Sigma Aldrich of analytical grade. Activated charcoal, isopropanol, ammonium acetate, chloroform, iso-amylalcohol and ethanol were used in the study.

DNA extraction method

The 25 mg of dried and powdered plant material of was soaked in 1.5 ml extraction buffer [120 mM Tris-HCl (pH 8), 1 M Sodium chloride, 50 mM EDTA (pH 8), 0.5M Sucrose, 2% Triton X and β-Mercaptoethanol-0.2 % (v/v) along with 0.5 % (w/v) of activated charcoal] for a period of an hour at room temperature and later the soaked material was homogenized uniformly in pestle and mortar. The homogenized material was transferred into a micro-centrifuge tube and centrifuged at 15000 rpm for 15 minutes. The supernatant phase was transferred into a new micro-centrifuge tube and vortexed with an equal volume of solvent mixture (chloroform and Isoamly alcohol, 24:1v/v). The sample mixture was again centrifuged at 15000 rpm for 10 minutes,

to obtain uniform and vigorous mixing. The centrifuged solution was mixed with 0.7ml of isopropanol and the mixture was incubated for 30min at room temperature. The incubated solution was then centrifuged at 4ºC for 10 minutes at 15,000 rpm to obtain DNA pellet precipitation. The DNA pellet was washed twice with washing buffer (15mM ammonium acetate in 75 % (v/v) ethanol) and allowed to air dry at room temperature. The resultant DNA was dissolved in TE buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)] and stored at 4°C refrigerated condition.

Quantification of DNA

It was determined by measuring the ratio between UV absorbance at 260 nm and at 280 nm using a Nano Photometer (Thermo Scientific™ Nano Drop 2000). If the measured *A*260/*A*280 ratio falls between 1.7–1.9, the isolated DNA was considered to be pure and free of impurities. Further, the DNA was observed on one percent agarose gel by electrophoresis using 1X TBE buffer. The DNA fragments were stained with ethidium bromide and viewed under UV light (Gel doc XR^+ System) and subsequently photographed. The isolated DNA was further used for PCR amplification.

PCR amplification

PCR amplification was carried out in 25 µl volume containing 100 ng of template DNA, 2 mM $MgCl_2$, 10 pmol of primer ITS1 and ITS4, 2.5 µl of 10X assay buffer [Assay buffer:50 mM KCl, 1.5 mM $MgCl_2$ and, 0.2 mM each of dNTPs and 1 unit of Taq DNA Polymerase]. The PCR programme had 35 cycles, in which the first denaturation was carried out at 94°C for 3 minutes, segment denaturation at 94°C for 1 minute, annealing at 35.5°C for 1 minute, extension at 72°C for 2 minutes and final extension at 72°C for 3 minutes. The amplifications were carried out in DNA thermal cycler (Eppendorf, Germany). The amplified products were loaded onto one percent agarose gel stained with ethidium bromide and documented in Gel doc unit (Gel doc XR^+ System).

ISSR analysis

PCR amplification of template DNA using a 3′ anchored ISSR primer was carried out in a volume of 25 µl, containing 100 ng of template DNA, 2 mM $MgCl_2$ 10 pmol of primer ITS1 and ITS4, 2.5 µl of 10X assay buffer [50 mM KCl, 1.5 mM MgCl2, and, 0.2 mM each of dNTPs and 1 unit of Taq DNA Polymerase].

The PCR amplification was carried out on an Eppendorf Mastercycler-Gradient thermal cycler and which includes a cycle of 4 minutes at 94ºC, 1 minute at 45ºC and 1 minute at 72ºC, 45 cycles of 1 minute at 94ºC, 1 minute at 50ºC and 1 minute at 72ºC followed by a final incubation at 72ºC for 10 minutes. The PCR products obtained by AP-PCR and ISSR were separated on two percent agarose gel by electrophoresis using 1X TBE buffer. The DNA fragments were stained with ethidium bromide and viewed under UV light and subsequently photographed.

Results and discussion

Medicinal plants are being used extensively in Ayurveda, Unani and Homoeopathic medicine. There is increased demand in the international market at the same time adulteration of the material with spurious material is becoming serious concern. The molecular authentication of the medicinal herbs is utmost important to maintain efficacy and therapeutic property of these plants. As the botanical medicines available in the dried form, the authentication using molecular methods is troublesome due to the quality of DNA isolated using the available extraction protocols. The isolation of DNA from dried material is complicated due to the presence of polysaccharides and polyphenolic compounds (Hanania *et al*., 2004) [22]. Presence of proteins in the reactions leads to inhibition of PCR (Saiki R 1990) [23]. Majority of the DNA extraction methods published, require a large amount of plant tissue homogenized in liquid nitrogen (Busconi *et al.*, 2003) [24]. The methods used for the isolation of DNA from dried material resulted in low DNA yield, sticky and viscous poor-quality DNA. (Do and Adams 1991) [25]. Further, the DNA obtained is not suitable for downstream processing like DNA barcoding, RAPD and ISSR (Weishing *et al*., 1995) [9, 26]. It is well illustrated that, the DNA isolated from above methods did not produce distinct bands and it was not suitable for downstream processing.

DNA obtained from dried material using activated charcoal, sorbitol, Phenol, RNase A and liquid nitrogen in extraction buffer. However, the requirement of expensive extraction chemicals and reagents were found limitations to the method in Krizman *et al.* (2006) [20]. The modifications adopted to this method of DNA extraction protocol explained in like removal of CTAB, addition of β-Mercaptoethanol along with Triton X the addition of high concentration of β-mercaptoethanol significantly improved the quality DNA extraction. The use of activated charcoal helps in removing interference of complex polysaccharides and polyphenols. The isolated genomic DNA from the dried plant material showed the significant difference in the quality and yield of nucleic acids. The purity of the isolated DNA was determined by measuring the absorbance ratio at 260 and 280 nm in Nano Photometer. The A260/A2800 ratio of 1.80 and 1.90 was recorded for the isolated DNA samples. The average significant yield of DNA, 1000 µg/µl (Nano Photometer) was obtained with the identified method (Table 1). The isolated quality DNA was amplified in PCR to obtain the expected product of 1.5 kb. The resulted DNA can be stored for long term and found suitable for RAPD, RFLP, ISSR and amplification of plant barcoding genes (ITS, *matk* and *rbcL* etc.). In the present study all the samples amplified PCR product from 500-800 bp of ITS gene (Fig. 1). The modifications adopted in the DNA extraction protocol allows the isolation of DNA from diverse plant species with good yield and purity compared to other available techniques. The isolated DNA is very much suitable for further PCR and ISSR analysis Acknowledgment

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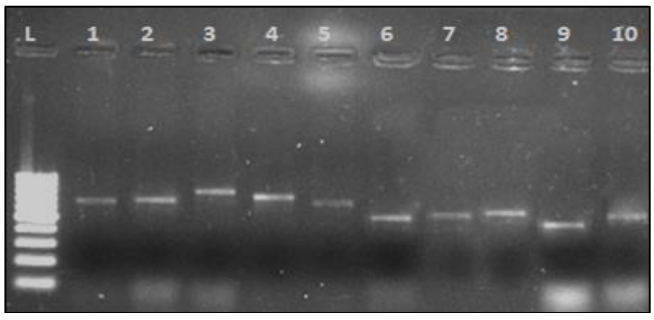
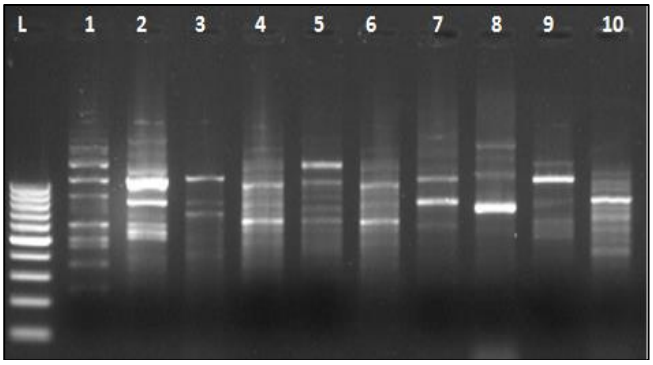


L. 1kb ladder, 1. E. alba, 2. A. Indica, 3. L. aspera, 4. T. arjuna, 5. P amarus, 6. T. cordifolia, 7. A marmelos 8. C. longa, 9. W. somnifera, 10. P. nigrum.

Fig 1: PCR Amplification of internal transcribed spacer (ITS) gene



L. 1kb ladder, 1. *E. alba*. 2. *A. Indica*, 3. *L. aspera* 4. *T. arjuna*, 5. *P amarus*, 6. *T. cordifolia*, 7. *A marmelos* 8. *C. longa*, 9. *W. somnifera*, 10. *P. nigrum*

Fig 2: ISSR profile generated by the primer ISSR-03

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