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Original Article

RAPD Analysis for Determination of Components in Herbal Medicine

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In this study, the RAPD (Random Amplified Polymorphic DNA) technique was employed for determination of the components in an Ayurvedic herbal prescription, *Rasayana Churna*. One-hundred-and-twenty decamer oligonucleotide primers were screened in the RAPD analysis to identify three Ayurvedic medicines, dried stem of *Tinospora cordifolia*, dried fruit of *Emblica officinalis* and dried fruit of *Tribulus terestris*, the Ayurvedic prescription. Primer OPC-6 simultaneously generated three distinct amplicons, each specific to one component. The marker with 600 bp is specific to *Tinospora cordifolia*; the marker 500 bp is specific to *Emblica officinalis* and the remaining marker >1000 bp was present in *Tribulus terestris*. Presence of three herbal medicines was determined when RAPD reaction with OPC-6 was performed. The technique was proved to contribute to the identification of components in Ayurvedic herbal preparation and thus helping to serve as a complementary tool for quality control.

Keywords: DNA fingerprinting – herbal medicine – RAPD – standardization

Introduction

Herbal medicine has been enjoying renaissance among the customers throughout the world. However, one of the impediments in the acceptance of these formulations is the lack of standardization and quality control profiles. Due to the complex nature and inherent variability of the chemical constituents of plant-based drugs, it is difficult to establish quality control parameters (1).

Rasayana churna is an Ayurvedic polyherbal formulation used for its antioxidant, immunomodulatory and rejuvenating purpose. It includes three herbal drugs, dried stem of *Tinospora cordifolia* (family: Menispermiaceae), dried fruit of *Emblica officinalis* (family:Euphobiaceae) and dried fruit of *Tribulus terestris* (family:Zygophylaceae), in powder form (2). All three plants are widely used as adaptogenic and in Rasayana therapy. Our major objective therefore, was to develop molecular tools for accurate identification of different components present in herbal formulations. Random Amplified Polymorphic DNA (RAPD) involves the use of a single 'arbitrary' primer in a polymerase chain reaction (PCR) and results in the amplification of several discrete DNA products (3). Each product is derived from a region of the genome that contains two short segments in inverted orientation, on opposite strands that are complementary to the primer and sufficiently close together for the amplification to work (4).

Several herbal drugs on the market still cannot be identified based on their morphological or histological characteristics. The present study aimed to detect the presence of various herbal ingredients in *Rasayana churna* formulation. After 120 primers were screened; primer OPA-6 was selected to simultaneously identify *T. cordifolia, E. officinalis* and *T. terestris* in Ayurvedic prescription. The assay will be helpful in quality control of herbal medicines in the market.

Materials and Methods

Rasayana churna was procured from the local market of Pune. Dried stem of *T. cordifolia*, dried fruit of

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E. officinalis and dried fruit of *T. terestris* were collected from the Pune regions of India. The samples were authenticated and voucher specimens were deposited in our Pharmacognosy and Phytochemistry department.

Reagents and chemicals

CTAB (20% [w/v]), 1 M Tris–Cl (pH 8), 5 M EDTA (pH 8), 5 M NaCl, 3 M sodium acetate, ethanol (AR grade), chloroform-IAA (24:1 [v/v]), polyvinylpyrrolidone (PVP) (~40 000 mol wt) (Sigma), β-mercaptoethanol. All the chemicals used in the experiments were of analytical grade.

DNA Extraction

Dried stem of *T. cordifolia*, fruit of *E. officinalis* and fruit of *T. terestris* were washed with 70% ethanol for 5 min and then with sterile deionized water for 1 min following ethanol, using sonication to avoid surface contamination. After being air-dried, the sample was cut into pieces and ground into a powder with liquid nitrogen using a mortar and pestle. Where as *Rasayana churna* was directly ground with liquid nitrogen. DNA was extracted from each of the samples using a modified CTAB (cetyltrimethylammonium bromide) procedure (5).

RAPD Reaction

PCR amplifications were tested with RAPD primers from commercially available kits (OPA, OPC and OPG) (Operon Technologies, CA and USA). One-hundred-and-twenty primers were screened for presence of distinct and consistent bands. On the basis of which, four primers (OPA-16, OPC-7, OPC-13 and OPG-5) were found useful. PCR reaction mixture consisted of buffer Tris–KCl (20 mM Tris–HCl, pH 8.4 and 50 mM KCl), 2 mM $MgCl_2$, 0.8 μM primer, 1 mM dNTP, 5 U/Reaction of *Taq* DNA polymerase (Gibco BRL), 15 ng template DNA and sterile deionized water to a total volume of 25 μl. For DNA amplification, Eppendorf 2400 DNA thermocycler was initially programmed for heating at 94°C for 5 min, followed by 45 cycles of 1 min at 94°C, 1min at 36°C and 2 min at 72°C. Immediately after the last amplification cycle, reaction mixture was kept at 72°C for 5 min and then cooled at 4°C for 20 min. PCR amplified DNA fragments were separated by electrophoresis in agarose gel (1.5%) and stained with ethidium bromide (20 μg 100 ml^{-1}) at 3 V cm^{-1} for 4–5 h. A sample without template DNA was included as a negative control in each experiment to check contamination. Electrophoretic profile was visualized under UV radiation and photographed with BIO-RAD. Sizes of DNA fragments were estimated by comparison with standard Ladder 100 bp (Gibco BRL). Electrophoretic profiles were analyzed for polymorphism based on the presence and absence of DNA bands on agarose gel.

Results and Discussion

According to WHO general guidelines for methodologies on research and evaluation of traditional medicines, first step in assuring quality, safety and efficacy of traditional medicines is correct identification. In the present article *Rasayana churna*, an Ayurvedic formulation was chosen to test the reliability of the quality control and presence of three herbal drugs in it, using RAPD technique. The prescription contains three ayurvedic herbal medicines *T. cordifolia, E. officinalis* and *T. terestris*. RAPD technique has been used for determination of different components present in herbal formulation (6,7).

For DNA extraction we required modified protocol especially in the case of *E. officinalis*, because of its highly acidic nature. When DNA was isolated using methods described by Doyle and Doyle (8) and Murray and Thompson (9), DNA of desired quality and quantity could not be isolated. But when extracted by the reported method of Warude *et al.* this gave good quality, high-molecular-weight DNA yield that is free of contaminants and colored pigments. Extraction of DNA from Churna was a critical step as Churnas are finely powdered resulting in destruction of tissue structure. In case of *Rasayana churna* CTAB protocol gave very low yield. The DNA was amplified in PCR but lacked all bands, so DNA was extracted by using a kit, [DNeasy plant Mini Kit (50) Qiagen]. For RAPD reaction, it was necessary to standardize the following variables for successful amplification using PCR. RAPD amplification is not reproducible below a certain concentration of genomic DNA and produces 'smears' or results in poor resolution if DNA concentration is high. So series of dilutions were made to check good amplification. PCR trials were undertaken with different concentrations of $MgCl_2$ (0.5 mM, 1 mM 1.5 mM) keeping all other parameters constant. $MgCl_2$ of 1.5 mM concentration was proved best in 25 μl reaction volume. For most amplification reactions 0.5–1.5 units of the enzyme were used. Initially amplification reactions were carried out with 0.9 units of *Taq* polymerase, however, this was not found to work. Therefore the quantity was reduced to 0.6 units of *Taq* polymerase per 25 μl reaction volume, which gave good results. Primer annealing in most reactions was carried out at 36°C to 55°C. In all PCR trials the annealing temperature of 36°C has been used which was determined by several trials of PCR. Different concentrations of dNTP ranging from 100 μM to 250 μM of each dNTP per 25 μl reaction volume were tried. Finally 100 μM of each dNTP proved the best. DNA denaturation is a critical step in DNA amplification reactions. For most DNA amplification reactions incubation time for DNA denaturation is 30 s to 1 min at 92°C to 95°C. To assure that all long DNA molecules have denatured, denaturation was carried out at 94°C for 1 min.

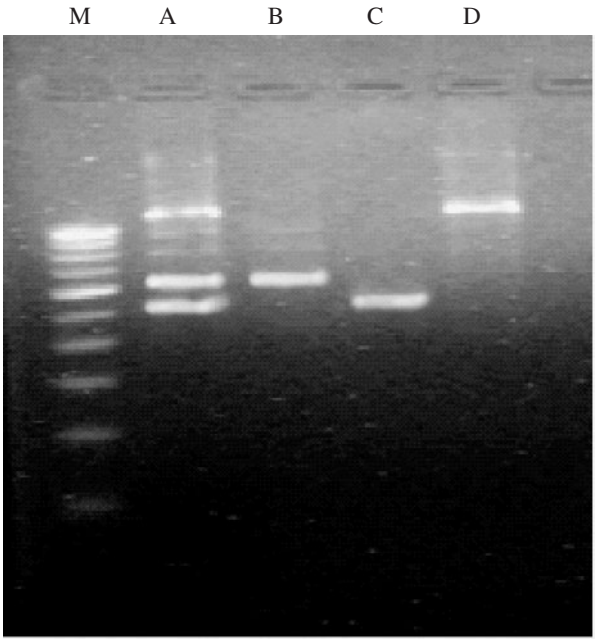

Figure 1. RAPD fingerprints of the samples used in the study generated by primer OPC-6. M-100 bp molecular weight marker; A- *Rasayana Churna*; B- *T. cordifolia*; C- *E. officinalis*; D- *T. terestris*.

This technique has frequently been used for the detection of genetic variability in plants. The advantages of the approach are its rapidity, simplicity and the absence of any need for prior genetic information of the plant. RAPD fingerprints patterns obtained are consistent irrespective of the plant source or age (10,11). These characters are especially advantageous for the identification of herbal medicine because little DNA exist in the dried material and also because sequence data are difficult to obtain. PCR amplifications were tested with RAPD primers from kits OPA, OPC and OPG (Operon Technologies, CA and USA). Thus RAPD markers, which are specific to *T. cordifolia, E. officinalis* and *T. terestris*, were obtained through the primers screening. The primers giving distinct bands were selected for further study. Primer OPC-6 clearly differentiates all components of *Rasayana Churna* and can be used for simultaneous determination of components (Fig. 1). The study reported that the RAPD technique was applied to the identification of the components in an Ayurvedic prescription. Accordingly, an efficient, precise and sensitive method for identifying components for *Churnas* has been established and will contribute significantly in quality control. Significance of present work is that single primer can differentiate three different components in single run. More high quality-linked DNA markers of the herbal medicines will be developed in further studies, which can provide an alternative tool to monitor the quality of herbal medicines.

Conclusion

We established DNA fingerprinting profile using RAPD markers, for the simultaneous identification and quantification of *T. cordifolia, E. officinalis* and *T. terestris* in *Rasayana churna*. Primer OPC-6 clearly differentiates all components of *Rasayana churna*. Accordingly, an efficient, precise and sensitive method for identifying components in *Churnas* has been established and will contribute significantly in quality control.

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