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Optimization of DNA extraction from seeds and leaf tissues of Chrysanthemum (*Chrysanthemum indicum)* for polymerase chain reaction

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Abstract:
Chrysanthemums constitute approximately 30 species of perennial flowering plants, belonging to the family Asteraceae, native to Asia and Northeastern Europe. Chrysanthemum is a natural cosmetic additive extracted from Chinese herb by modern biochemical technology. It has the properties of anti-bacterial, anti-viral, reducing (detoxification) and anti-inflammation. It possesses antioxidant characteristics, which could assist in minimizing free-radical induced damage. Therefore, it is widely used in skin and hair care products. Chemical composition of this herbal remedy includes kikkanols, sesquiterpenes, flavonoids, various essential oils containing camphor, cineole, sabinol, borneole and other elements that interfere with DNA, causing erroneous or no PCR products. In the present study, testing and modification of various standard protocols for isolation of high-quality DNA from leaf tissues and seeds of *C. indicum* was done. It was observed that the DNA obtained from seeds and leaf tissues with a modified cetyltrimethylammonium bromide buffer protocol was of good quality, with no colored pigments and contaminants. Also, DNA could be extracted from leaf tissues without using liquid nitrogen. Quality of DNA extracted from seeds was much better as compared to that extracted from leaf tissues. The extracted DNA was successfully amplified by PCR using arbitrary RAPD primers. The same protocol will probably be useful for extraction of high-molecular weight DNA from other plant materials containing large amounts of secondary metabolites and essential oils.

Key Words: Cetyl trimethyl ammonium bromide, PCR Amplification, secondary metabolites, EDTA, *Chrysanthemum indicum*

Background:
Chrysanthemums, often called mums or chrysanths, are of the genus (*Chrysanthemum*) constituting approximately 30 species of perennial which is native to Asia and northeastern Europe. Chrysanthemum plants exhibit potential medicinal activities as shown by earlier studies, e.g., *C. indicum* showed inhibitory action against bacteria and viruses **[1, 2].** *C. indicum* is a traditional herb, commonly used to treat various disorders, hypertension symptoms and several infectious diseases in Koreanand Chinese medicine **[3, 4].** Organic extracts of *C. coronarium* fresh flowerheads have shown antibacterial **[5],** insecticidal **[6]** and antiparasitic activities **[7, 8].** Pyrethrins, complex esters extracted from *C. cinerariaefolium* exhibited insecticidal effects **[9]** and in vitro activity against Herpes simplex virus **[10].** Recently, the use of *C. trifurcatum* flowerheads has been reported in Tunisian traditional medicine to treat constipation **[11].** Chrysanthemum tea has many medicinal uses, including an aid in recovery from influenza. Chrysanthemum plants have been shown to reduce indoor air pollution.

Currently, DNA analysis is the most desirable method for molecular characterization of species. Extraction of high quality DNA from medicinal plants and its purification is difficult because of the presence of large quantities of secondary metabolites, poly-saccharides and proteins such as tannins, alkaloids, and polyphenols. These compounds degrade the quality of DNA by precipitating along with it, thus reducing yield **[12].** An array of DNA isolation protocols have been optimized and were used in various combinations to isolate quality DNA from plants for analyses **[13-16].** Liquid nitrogen plays an important role in obtaining good quality DNA and hence, it has been used extensively for DNA extraction from fresh leaf and or other tissues. The objective of the present study was to standardize a protocol for obtaining high-quality DNA, suitable for PCR amplification without the use of liquid nitrogen for plant tissues including seeds of *Chrysanthemums* species.

Methodology:

Plant material

DNA was isolated from the leaves and seeds of *C. indicum,* which were collected upon 6-8 leaves stage. The young leaf tissues were maintained in ice and were crushed in the extraction buffer for DNA isolation. Seeds of *C. indicum*, collected from the farm grown plants were stored in zipper bags and maintained in the laboratory. Three protocols were followed for DNA isolation from *C. indicum*: isoamylalcohol, cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS). A modified CTAB buffer method was employed for post extraction analyses of DNA from seeds and leaf tissues.

***Reagents Employed*:**

(1) M Tris-C1 (pH 8.0, 9.5), 0.5 M EDTA (pH 8), 5.0 M NaCl; 3.0M sodium acetate (pH 5.2), CTAB (20%), chloroform:isoamyl alcohol (24:1, v/v), β-mercaptoethanol (AR grade); (2) Modified CTAB extraction buffer: 0.1 M Tris-Cl (pH 9.5), 20 mM EDTA (pH 8), 1.4 M NaCl, CTAB (2%, w/v), β-mercaptoethanol (1%, v/v) (added to the buffer just before use); (3) Pure cold (-20°C) isopropanol; (4) 70% ethanol; (5) Absolute ethanol; (6) TE buffer: 10 mM Tris-Cl buffer (pH 8.0), 1 mM EDTA (pH 8.0); (7) Enzyme: Taq DNA polymerase (Fermentas Inc.), Rnase a (Fermentas Inc.); (8) Buffer: Taq DNA polymerase buffer (Fermentas Inc.); (9) Nucleotides: dNTPs (G, A, T, C) and RAPD primers (GL A1-10); (10)TBE 5X: 54 g Tris, 27.5 g boric acid, 20 mL 0.5 M EDTA; (11) Agarose gel; (12) Ethidium bromide.

DNA extraction

(1) Fresh-leaf tissue (0.2 g) was ground in a 1.5-ml Eppendorf tube with a micro pestle and preheated freshly prepared 800 µL extraction buffers was immediately added to the tube; (2) Leaf tissues (0.1 g) were grounded to a fine powder with pestle and mortar and transferred to a 1.5-mL Eppendorf tube in which 800 µL extraction buffers was added; (3) The tube was incubated at 65°C for 35-45 min, with inversion during incubation; (4) An equal volume of chloroform: isoamyl alcohol (24:1, v/v) was added and the tubes were inverted thoroughly; (5) The tubes were centrifuged at 13,000 rpm for 15 min; (6) The supernatant was transferred to a fresh Ependorf tube; (7) Precipitate formed was removed by repeating steps 4 – 6; (8) An equal volume of absolute ice-cold isopropanol was added; (9) The tubes were again centrifuged at 13,000 rpm for 10 min; (10) Supernatant was discarded and the pellet was washed with 70% (v/v) ethanol; (11) The pellet was air-dried for 1 h at room temperature and then dissolved in 100 µL TE buffer; (12) RNase A [2 µL (1 µg/µL)] was added and the tubes were incubated at 37°C for 1 h; (13) For further purification, extraction with an equal volume of chloroform: isoamyl alcohol (24:1, v/v) was performed; (14) DNA was precipitated by adding 1/10th volume of 3 M sodium acetate, pH 5.2, and volume of ice-cold ethanol; the tube was inverted gently and this mixture maintained for 30 min at -20°C. It was then centrifuged at 13,000 rpm for 10 min; (15) Supernatant was discarded and the pellet was washed with 70% (v/v) ethanol; (16) The pellet was dried and dissolved in 100 µL TE buffer; (17) DNA concentration was measured by reading absorbance at 260 nm and by running aliquots on 0.8% agarose gel; (18) The DNA samples were stored at -20°C until further use.

PCR amplification and gel electrophoresis

PCR-based amplification of the purified DNA was carried out in a 20L-reaction mixture. The reaction mixture contained the following: 25 µg template DNA, 0.125-U *Taq* DNA polymerase, 1.6 mM dNTPs, 3.75 mM MgCl2, 1X *Taq* DNA polymerase buffer **[14]** and 2 mM Primer (GL, USA). Amplification of the DNA was performed in a thermocycler in the following manner: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The reaction mixture was stored at 4°C, until it was loaded onto the gel. The PCR products were fractionated on 0.8% agarose gel using 1X TBE buffer containing 10 mg/ml ethidium bromide and were visualized under UV light. The gel was photographed using the UV gel documentation system **[17].**

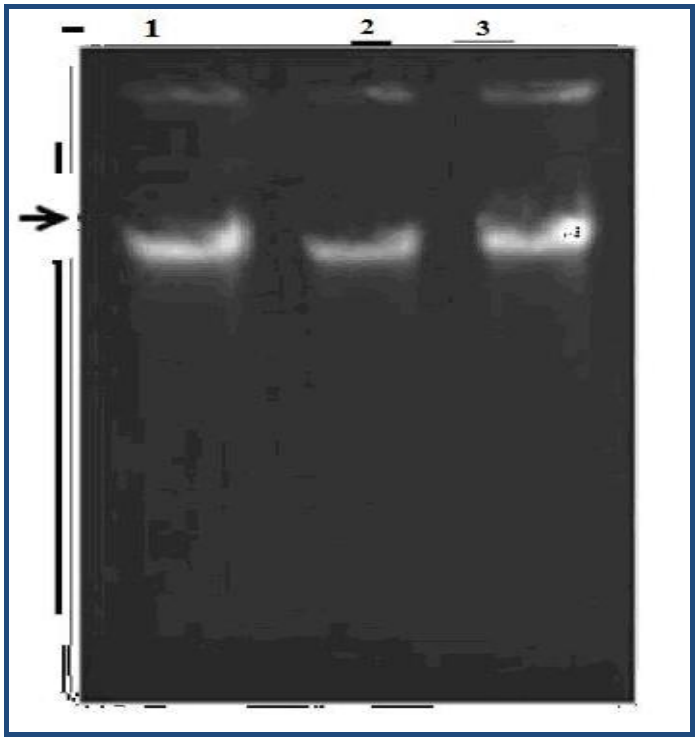

Figure 1: Bands obtained after agarose gel electrophoresis showing total DNA isolated from three samples of leaf tissue of *Chrysanthemum indicum* (lanes 1, 2 and 3).

Discussion:

Previous study has shown the successful isolation of high quality DNA from various plant parts **[18, 19].** The major aim of the present study was to optimize a rapid and inexpensive protocol for DNA isolation yielding high quality DNA. Hence, three different protocols using Isoamyl alcohol, CTAB, and SDS respectively were employed to isolate and analyze DNA from different plant parts of *C. indicum.* The DNA obtained showed very faint bands upon gel electrophoresis due to the presence of higher levels of secondary metabolites in *C. indicum* such as polysaccharides, polyphenols, flavonoids, and essential oils, which may get co-precipitated with DNA, thus interfering with enzymatic analysis. No amplification products were seen after PCR analysis. However, the results from the modified CTAB buffer method gave better results than the other tested protocols.

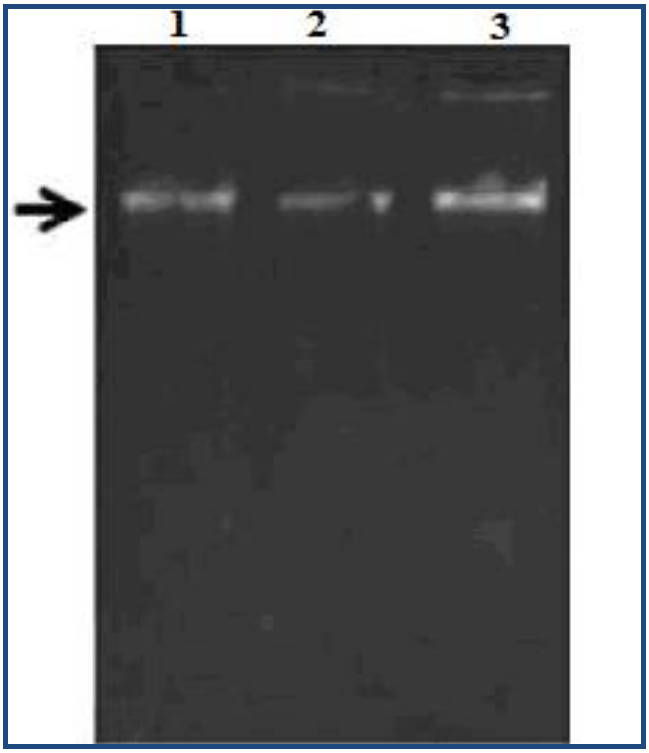

Figure 2: Bands obtained after agarose gel electrophoresis showing total DNA isolated from three samples of seeds of Chrysanthemum indicum (lanes 1, 2 and 3).

The DNA concentration was measured as ratio of absorbances, i.e., A_{260}/A_{280} and was found to be 1.5, indicating high levels of contaminated proteins and polysaccharides. Total DNA isolated from leaves and seeds of *C indicum* was analysed by agarose gel electrophoresis **(Figures 1 & 2).** The modified protocol thus yielded DNA of high purity as indicated by the ratio of absorbance (A_{260}/A_{280}), which was 1.75 indicating DNA frees from essential oils, polyphenols, flavonoides, and polysaccharides. The high quality DNA obtained from the modified protocol was suitable for PCR and showed high intensity amplification with arbitrary RAPD primers **(Figure 3).** PCR amplification also indicates that the DNA was free from interfering compounds, and it would be suitable for other DNA characterization techniques such as restriction fragment length polymorphism (RFLP).

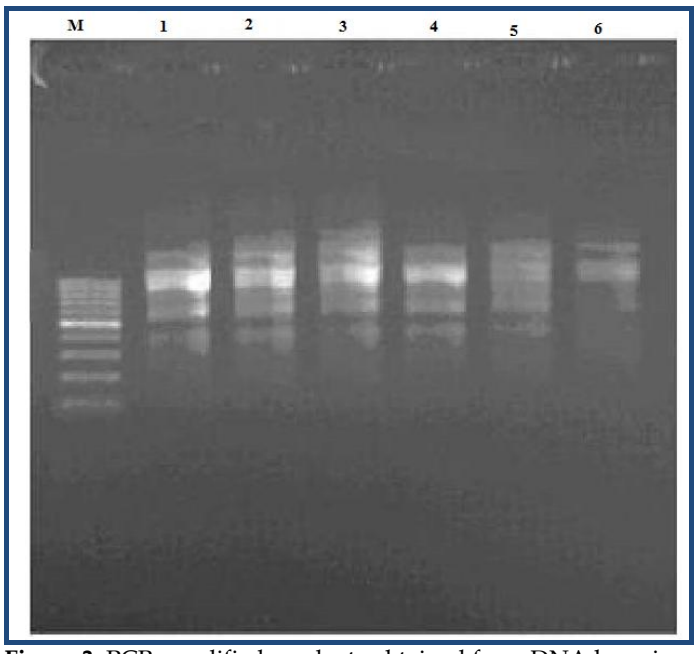

Figure 3: PCR-amplified products obtained from DNA by using random primers. M: 100-bp DNA ladder; lanes 1, 2, and 3: Chrysanthemum indicum leaf tissue DNA amplified with primer GL A-03; lanes 4, 5, and 6: *C. indicum* seed tissue DNA amplified with primer GL A-03.

DNA isolation is a primary and critical step for molecular analysis of any plant species. The chemicals routinely used in many protocols are too expensive to be used for routine DNA extraction. Therefore, it was necessary to establish an inexpensive and less time-consuming protocol for DNA extraction from various plant parts. *C. indicum* is one of such plant species which contains high amounts of secondary metabolites and essential oils. Therefore the extraction of genomic DNA from this plant is difficult. The modified CTAB buffer containing Polyvinylpyrrolidone (PVP), a compound known to suppress polyphenolic oxidation, was also employed to extract DNA from *C. indicum* using liquid nitrogen **[20].** However, PVP has been used frequently in CTAB extraction protocols **[14]** and prevents contamination of DNA **[21].** However, the use of PVP in CTAB buffer did not improve the yield or quality; rather we obtained significantly better results without its use in DNA extraction. We also tested seeds for extraction of DNA because *C. indicum* is an annual medicinal species and seeds are easily preserved in plastic bags for a long time without specific preserving conditions. High-quality DNA was obtained from seeds or fresh leaves of *C. indicum* for post-extraction enzymatic assay. We anticipate that this protocol will be adequate for extracting high-molecular weight DNA from other species containing large amounts of secondary metabolites and essential oils.

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