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Annexures

Herbarium Certificate

भारत सरकार
GOVERNMENT OF INDIA
पर्यावरण, वन एवं जलवायु परिवर्तन मंत्रालय
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सं0 भावस / उक्षेके / No. : BSI/NRC /Tech./Herb(Ident.)/2022-23/328

दिनांक / Dated 07.2022

प्रमाणपत्र / CERTIFICATE

प्रमाणित किया जाता है कि कु0/श्रीमति **अंजली उनियाल, स्वामी रामा हिमालयन विश्वविद्यालय देहरादून 248016, उत्तराखंड** से प्राप्त निम्न पादप नमूना निम्नानुसार (वानस्पतिक नाम) इस कार्यालय के विद्यार्थी पादपालय में परिग्रहण **किया गया है ।**

Certified that the following plant sample received from **Ms. Anjali Uniyal, Swami Rama Himalyan University, Jolly Grant**, Dehradun 248016, Uttarakhand is accessioned at herbarium (BSD Student Herbarium) of this office:

पादप का नाम / Plant name	कुल / Family	परिग्रहण सं. / Acc. No.
1. ***Rheum webbianum*** **Royle** Syn: *Rheum emodi* Wall.	Polygonaceae	1146

29/07/22

(एस.के. सिंह / S.K.Singh)
(वैज्ञानिक–ई / कार्यालयाध्यक्ष / Scientist-E/HoO)

Publications

Paper Publications

- **Uniyal, A.,** Kumar, A., Kumar, V., Gupta, S. and Kumar, V. (2023). An effective and low-cost method for DNA extraction from *Rheum* species (A medicinal herb) without using liquid nitrogen for RAPD/ISSR Study. Research Journal of Biotechnology, 18(9):170-174.
- **Uniyal, A.,** Kumar A., Upadhyay, S., Kumar, V., Gupta, S. (2021). Assessment of genetic diversity of *Rheum species* (Endangered medicinal herb of Indian Himalayan region) using molecular markers. Research Journal of Biotechnology, 16(11): 147-154.

Conferences Attended

- "An effective and low cost method for DNA extraction from the leaves of *Rheum species* (An endangered medicinal herb of Indian Himalayan region)" In 16th Uttarakhand State Science and Technology Congress (2020-2022); Authors; Anjali Uniyal, Sweta Upadhyay, Dr Akhilesh Kumar, Dr Sanjay Gupta.
- "Genetic Diversity Assessment Using Molecular Markers for the Germplasm Conservation of *Rheum Species"* in International Conference, "Ganga Ayur Con 2022", Held at Gurukul Campus Haridwar, Organised by Uttarakhand Ayurvedic University; Authors; Anjali Uniyal, Sweta Upadhyay, Dr Akhilesh Kumar, Dr Sanjay Gupta.
- " Evaluation of In-Vitro Antimicrobial Activity of Plant Extracts of *Rheum* Species Against Bacterial and Fungal Strains" At an International Conference (Biosangam- 2022), Organised by Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, Pryagraj-India; Authors: Anjali Uniyal*, Akhilesh Kumar, Sweta Upadhyay, Sanjay Gupta and Vijay Kumar.

An effective and low-cost method for DNA extraction from *Rheum* species (A medicinal herb) without using liquid nitrogen for RAPD/ISSR Study

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Abstract

Rheum is an important medicinal plant belonging to family Polygonaceae and is found to have anti-microbial, anti-purgative and anti-cancer properties. The objective of the present study is to standardize an effective and low-cost method for the extraction of DNA from the young juvenile leaves of Rheum plant obtained from the Uttarakhand region. The protocol involved the extraction of DNA using a modified CTAB method without the use of any liquid nitrogen. The concentration of NaCl used in extraction buffer was slightly higher (1.4M) to remove polysaccharides and varying ratios of Beta-mercaptoethanol used to remove the secondary metabolites and keep them away from interfering the extraction process.

The absorbance ratio of the extracted DNA at $A_{260/280}$ nm was within the permissible range of 1.7-2.0, indicating the purity of DNA and showing that the DNA is contamination free. Its concentration was approximately 1485 ng/µl and it was found suitable for molecular-based study.

Keywords: *Rheum*, CTAB, DNA extraction, liquid nitrogen, RAPD, ISSR.

Introduction

Rheum species is a perennial herbaceous plant (generally known as Rhubarb) belonging to family Polygonaceae. Genus *Rheum* consists of a total of 60 species found around the world out of which only 10 species occur in the Indian Himalayan region. It is frequently used as medicinal plant usually called as the "Wondrous drug" as it possesses a lot of therapeutic actions. They are pharmacologically important plants possessing diuretic, anti-cancer, anti-bacterial, anti-tumour, anti-fungal, haemostatic and antihypertensive properties. They lower serum cholesterol and possess anti-inflammatory properties. A lot of secondary compounds are isolated from *rheum* plant responsible for varied activities of plant. In, India, a country lying in tropical zone is a cradle for many species of medicinal plant. But due to the over exploitation by the pharma industries, the medicinal plants are on the verge of depletion.

The plant biotechnology offers alternative to conventional plant improvement techniques, most of which have application to the urgent problems of the improvement of forest crop in developing countries. The isolation of high-quality DNA is very important for molecular analysis. The DNA isolation in plant is hampered because of the presence of secondary metabolites, polyphenols and presence of polysaccharides that interfere with the extraction procedure[9].

So, there is always a need to develop protocols for the isolation of good quality and higher yields of DNA that are also cost friendly. Many factors lead to degradation of DNA during isolation. The presence of polysaccharides is another factor characterised by the presence of viscous liquid in aqueous phase[4]. The polyphenols are the major problems in *rheum* species that impart brown colour to the pellet, thereby leading to poor quality of DNA.

Various kits have also been used for the isolation of DNA that includes the use of toxic and hazardous chemicals. They are not so pocket friendly and require high profile laboratory containment areas[3,12]. The present study focuses on providing an efficient, easy and low-cost method for the isolation of high quality and high yield of DNA from *Rheum* species.

Material and Methods

Plant material: For the extraction of genomic DNA, fresh juvenile leaves of *Rheum spp.* were collected from Chopta region, District Rudrapryag, Uttarakhand (30.3462° N, 79.0485° E). A minimum of 10 accessions was taken for DNA isolation. The leaves were stored at -20°C till further use to prevent damage and bruising.

Solutions and Reagents

i. CTAB Buffer (Cetyl-trimethylammoniumBromide): (Preheated at 65°C) 100 mM Tris-HCl (pH-8.0), 20 mM EDTA (Ethylenediaminetetraacetic acid) (pH-8.0), 1.5 M NaCl, 2% CTAB, 2% polyvinylpyrollidone (PVP), 5 mM Ascorbic acid and 0.3% β-mercaptoethanol.
ii. 5 M Amonium acetate solution
iii. Chloroform: isoamyl alcohol (24:1)
iv. Ethanol 76% and 96 %
v. Isopropanol (chilled)
vi. TE Buffer: Tris HCl 10mM, EDTA 1mM pH 8.0
vii. Wash buffer: 998µl of 96% ethanol and 2µl of ammonium acetate

DNA Extraction Protocol (Figure 1)

i. Total genomic DNA was extracted from the young leaves of *Rheum* species using slightly modified protocol of Doyle and Doyle[2].

ii. Cut the leaf material into small pieces, sterilize using distilled water and then 70 % ethanol followed by distilled water again.
iii. 1.5gm of leaf sample was grinded in pre chilled mortar and pestle with 3 ml extraction buffer and 1 ml liquid sample. 3 µl β-mercaptoethanol was taken in 2 ml Eppendorf tube (Proper ice-cold conditions should be maintained to prevent DNA denaturation or degrading by nuclease activity. The appearance of brown colour indicates degradation of DNA).
iv. The suspension was vortexed for 15 seconds and incubated at 60 °C for 30 minutes.
v. Equal volume of chloroform: isoamayl alcohol (24:1) was added followed by inversion mixing of Eppendorf.
vi. Centrifuge at 15,000 rpm for 10 minutes.
vii. The supernatant (aqueous phase) was collected in fresh microfuge tube and equal volume of chilled isopropanol was added. The Eppendorf's were kept overnight at -20 °C to obtain a stable pellet and to allow maximum amount of DNA to be pelleted (The aqueous phase should be collected carefully to avoid any mechanical damage to DNA).
viii. Centrifuge at 10,000 rpm at 4 °C for 10 minutes to obtain pellet. Discard the supernatant without disturbing the pellet and wash the pellet with 998 µl of 76% ethanol and 2 µl of 10 mM ammonium acetate for at least 30 minutes.
ix. Centrifuge at 10,000 rpm for 5 minutes.
x. Wash the pellet in 500 µl of 96% ethanol, centrifuge at 10,000 rpm for 10 minutes and the supernatant is again discarded. The remaining pellet in the tubes is then dried in a vacuum drier/air. The pellet is resuspended in 100 µl of T.E buffer and is allowed to dissolve. The sample is then stored at 4°C.

Qualitative and Quantitative DNA Analysis: The yield and purity of extracted DNA were measured by using a UV-VIS spectrophotometer (Shimadzu) at 260 nm. DNA purity was estimated by taking the absorbance ratio A_{260}/A_{280}. Any contamination in the form of polysaccharides was assessed by measuring the absorbance at $A_{260/230}$[11]. For visualization, all the extracted DNA samples were run on 0.8% agarose gel and observed in gel documentation system (Figure 2).

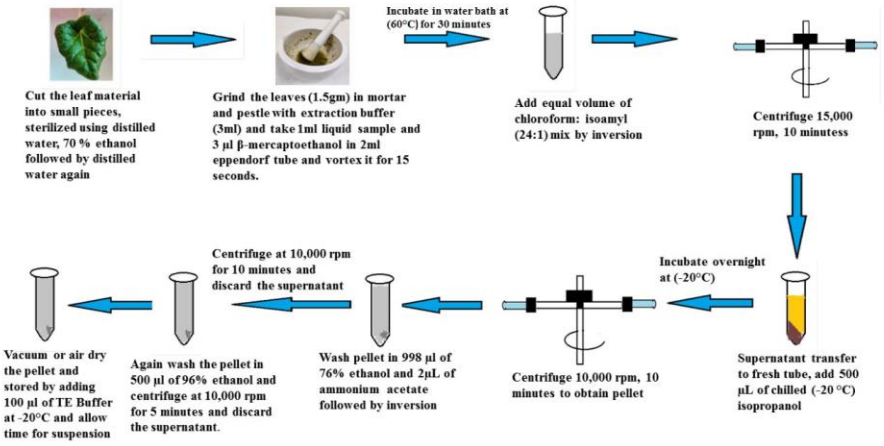


Figure 1: **Pictorial representation of modified CTAB DNA isolation Protocol**

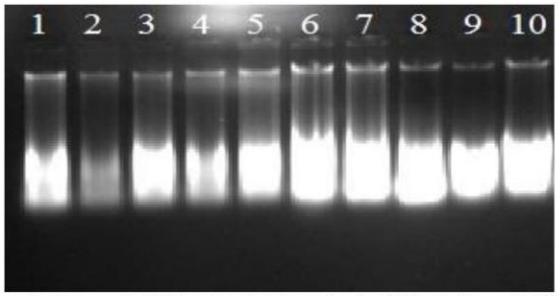


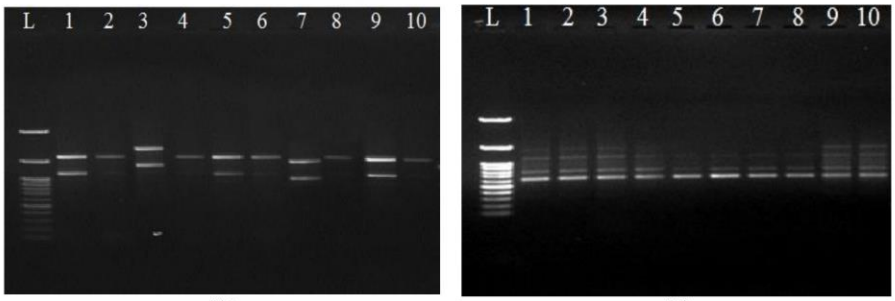
Figure 2: Gel photograph of genomic DNA extracted from ten individuals (Lane 1-10) leaves of *Rheum spp.* Gel photographs were resolved on 0.8% agarose gel

RAPD Analysis: The Random Amplified Polymorphic DNA analysis was carried out with two primers OPB 04 (GGACTGGAGT) and OPA19 (CAAACGTCGG) initially obtained from Sigma Aldrich[6].The PCR amplification was carried out in 15µl reaction mixture in a PCR (Table 1). The PCR amplification cycle consisted of: one cycle of denaturation at 95°C for 5 minutes, 40 cycles each of 94°C for 1 minute, 37°C for 1 minute and 72°C for 1 minute annealing and extension cycle, followed by final extension at 72°C for 8 minutes. The reaction mixture was kept at hold at 4°C till taken for storage. The PCR products were visualised on 1 % agarose gel and bands were observed in Gel documentation system (Figure 3).

ISSR Analysis: For ISSR-PCR analysis, UBC 836 (AGAGAGAGAGAGAGAGCTA) primers obtained from Sigma Aldrich were used[10]. The PCR amplification was carried out in 20µl reaction mixture in a thermal cycler (Table 2). The amplification cycle consisted of one cycle of denaturation at 94°C for 5 minutes, 40 cycles each of 94°C for 30 seconds, 49.3°C (annealing) for 30 seconds and 72°C (extension) for 1 minute, followed by final extension at 72°C for 10 minutes. The reaction mixture was kept at hold at 4°C till taken for storage. The PCR products were visualised on 1 % agarose gel and bands were observed in gel documentation system (Figure 4).

Table 1
PCR reaction mixture for RAPD

S.N.	Component	Concentration	1X
1.	Taq Buffer	10 X	1.5 µL
2.	$MgCl_2$	3mM	0.4 µL
3.	DNTPs	0.2 mM	1.2 µL
4.	Primer	0.2 µM	0.3 µL
5.	Taq Polymerase	1unit/µL	0.15 µL
6.	Template DNA	15-20 ng/ µL	2 µL in each vial
7.	Deionised Water		8.95 µL
Total Reaction Volume			**15 µL**



(A) **(B)**

Figure 3: RAPD Pattern (Lanes 1-10) obtained using primer (A) OPB 04 and (B) OPA 19, Bands are compared with 100bp DNA Ladder Lane L

Table 2
PCR reaction mixture for ISSR

S.N.	Component	Concentration	1X
1.	Taq Buffer	10 X	2 µL
2.	$MgCl_2$	2mM	0.8 µL
3.	DNTPs	0.2 mM	1.6 µL
4.	Primer	0.4 µM	0.8 µL
5.	Taq Polymerase	0.6 unit/µL	0.15 µL
6.	Template DNA	15-20 ng/ µL	2 µL in each vial
7.	Deionised Water		12.65 µL
Total Reaction Volume			20 µL

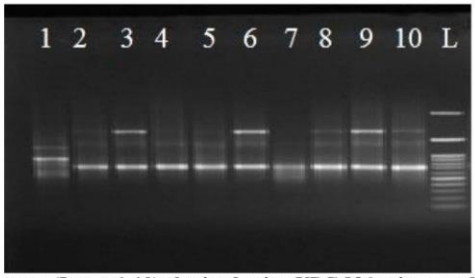


Figure 4: ISSR Banding patterns (Lanes 1-10) obtained using UBC 836 primer and bands were compared with 100bp DNA Ladder Lane L

Results and Discussion

The plant genomic extraction kits used does not provide with good yield and quality of DNA for *Rheum* species as evident by the presence of sticky polysaccharides and sheared bands in agarose gel[1].The researchers were facing difficulties to extract DNA because of colour produced in aqueous phase. Brownish pellet thus obtained indicated the presence of phenolic compounds. Hence, the present protocol for the isolation of DNA from *rheum* species by varying certain concentrations of NaCl, β-mercaptoethanol and PVP can be considered appropriate for isolation of DNA.

The DNA isolated from modified CTAB method of present study has varied applicability. The DNA concentration ranged from 1400-1600 ng/µl which in general indicates good yield. The use of prechilled mortar and pestle and frozen leaf sample prevented the damage of DNA and hence substituted the use of costly liquid nitrogen. The final DNA pellets so obtained were white in colour. Suman et al[8] have reported that high levels of β-mercaptoethanol remove the polyphenol leading to discoloration of pellet. So, in the present study, high levels of β-mercaptoethanol (0.3%) were used in order to obtain high quality of DNA[8]. NaCl when used in higher concentrations along with CTAB has been reported to remove polysaccharides[5].

In the present study, higher levels of NaCl have been used (1.5 M) that yielded high quality and greater yield of DNA apt for PCR amplification. The purity of extracted DNA can be depicted from the absorbance ratio at $A_{260/280}$ nm which was within the permissible range of 1.7-2.0 indicating the purity of DNA and showing that the DNA is contamination free and free from polyphenols and polysaccharides[7]. Clear banding patterns with discreet bands were also obtained in RAPD and ISSR analysis (Figures 3 and 4).

For the extraction of DNA from plants, basically young and juvenile leaves are the first preference as mature leaves contain high amounts of polyphenols and polysaccharides. However, sometimes it becomes difficult to obtain young leaves of certain species for molecular analysis. In that case, the present standardised protocol can be employed to obtain good quality of DNA. The present protocol can also be useful to obtain DNA from other plant species with higher levels of secondary metabolites and secondary components.

Conclusion

The present protocol describes a simple and cost-effective method for the isolation of DNA from *Rheum* Species. One of the major problems faced during isolation of DNA is the degradation of DNA by enzymatic actions or mechanical damages. The modified CTAB method of present protocol eliminates the use of liquid nitrogen and certain other costly and hazardous phenolic compounds, thereby making it cost efficient.

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in the International Conference (BioSangam 2022) during March 10 - 12, 2022 at the Department of Biotechnology, MNNIT Allahabad, Prayagraj, India.

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under the discipline

Biotechnology, Biochemistry and Microbiology

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