Chapter 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Selection of Sampling sites

The selection of sampling sites is a crucial step in any sampling process, as it determines the representativeness and accuracy of the results. A number of site visits and collection tours have been undertaken during 2021-2022 in the months of June-September covering diverse regions of Uttarakhand. The *Rheum* plant is available at different locations in Uttarakhand State, but the sampling site is selected in such a way that they capture the variability of the parameter across the study area and it should be accessible for collection of the samples.

According to the previous studies and knowledge about the study area, the Rudraprayag district of Uttarakhand region was selected for sampling due to accessibility of selection sites and it can provide valuable information regarding the plant *Rheum.* The juvenile leaves of *Rheum* plant were collected from different geographical areas. The various sampling sites available for sampling of rheum species are depicted in Figure 3.1.

3.1.2 Criteria for Sampling

For the present study the sampling was done using random sampling method as the objective is to obtain a representative sample of the population. The plant samples so collected from different locations were submitted to Botanical Survey of India, Northern Regional Centre Dehradun, to validate the representativeness of the sample to ensure that the results are reliable and accurate.

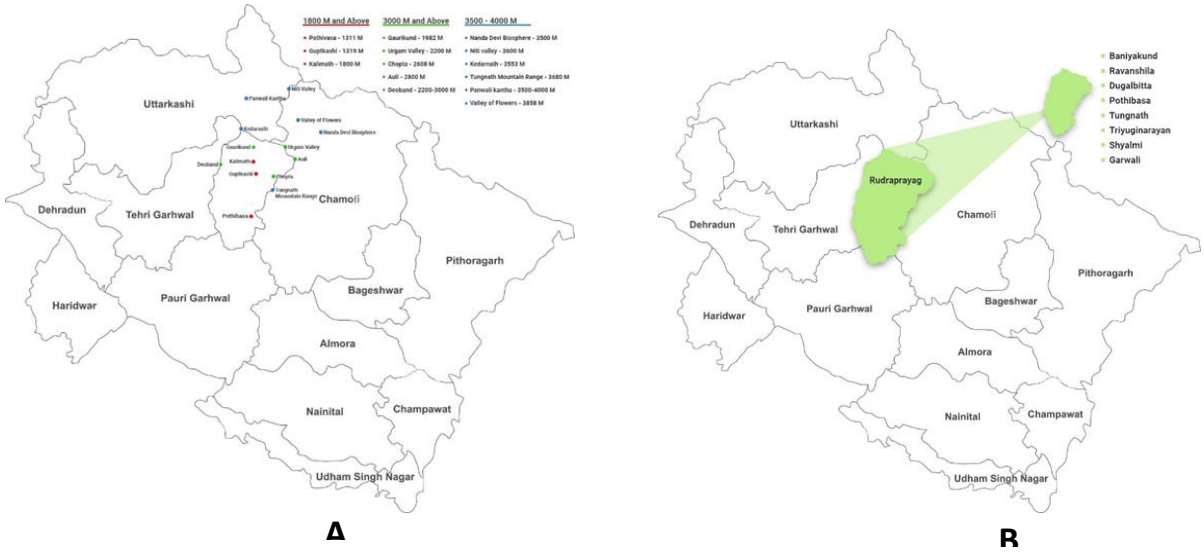


Fig 3.1: Sampling sites for *Rheum* species (A) In Uttarakhand, (B) Rudraprayag district

3.1.3 Collection of Plant Materials

During the period of July to September, leaves, rhizomes, and a few plants from various populations of *Rheum* were collected from three distinct regions of Uttarakhand. A total of 3 sampling sites were selected considering the climate, the soil type, and the surrounding vegetation. The months of July –September are best for sample collection as likewise other plants *rheum* also have specific growing seasons or flowering periods, and it's important to collect samples during these times.

For DNA analysis young and juvenile leaves of plant were collected separately (e.g. leaves, flowers, seeds, roots) by using sterilized tools to prevent contamination. For phytochemical analysis the mature leaves and rhizomes were collected and stored in separate sampling bags according to their sampling sites. The samples were then preserved properly by air drying, pressing, or freezing the plant material depending upon their use in the upcoming experimentation. The records of sampling process, the location, date, and method of collection were carefully noted on the sampling bags. Due to the threatened status of *Rheum* species, the collection of the plant, material was done judiciously. The small plantlets and the rhizome so obtained after sampling process were grown in Himalayan School of Biosciences, Swami Rama Himalayan University for further studies.

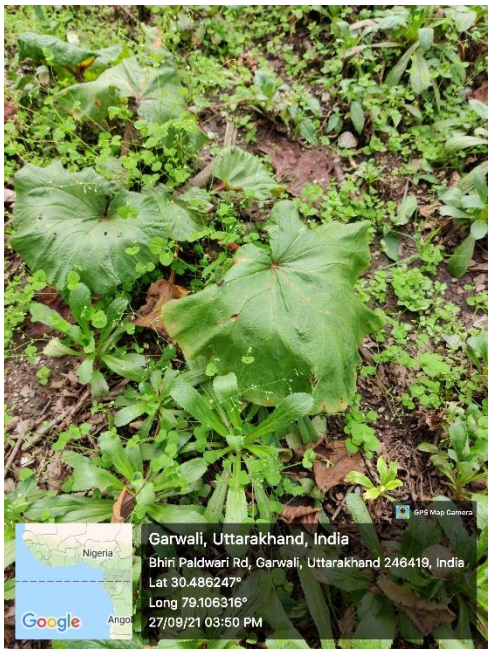
One copy of the plant was also submitted to Botanical Survey of India for authentication. A minimum of 12 accessions were selected for DNA isolation. The leaves were stored at -20°C till further use to prevent damage and bruising.

3.1.4 Details of Population Size

The fresh and juvenile leaves of *Rheum* plant were collected to carry on the present study (Figure 3.2). In total three different study areas were selected for sample collection. The study sites vary in the altitudinal variations and slight temperature variations. Three sampling sites comprising of alpine and temperate climate were selected (Table 3.1). The population 1, 2 and 3 with three populations size at slightly lower altitudes of 2200-meter comprising of Pothivasa, Triyuginarayan vicinity areas and Shyalmi village. Population 4, 5 and 6 comprise of sample populations from Tungnath, Ravanshila and Chopta (3600 meters) and population 7, 8 and 9 comprising of populations from Dugalbitta, Baniyakund and Garwali village (Average height 2400 meter). In total the collection of leaf samples of *Rheum* were done from nine different populations where each population consisted of 12 accessions from Rudraprayag district of Uttarakhand for evaluating the plant genetic diversity employing the use of molecular markers and phytochemical analysis of different plant samples of *Rheum* plant.

Table 3.1: Geographical details of populations of *Rheum* species

Population no.	Population Name	Population Code	Geographical details of Population		Elevation of population sites (Meter)	Sample size
			Longitude (E)	Latitude (N)		
Population 1	Pothivasa	PV	79.16º E	30. 28º N	2000	12
Population 2	Triyuginarayan	TN	78.54 79º E	30. 2645º N	2250	12
Population 3	Shyalmi	SM	79. 10 44º E	30. 30 24º N	2260	12
Population 4	Tungnath	TG	79.21699º E	30.48940º N	3600	12
Population 5	Ravanshila	RS	79.21070º E	30.48546º N	4000	12
Population 6	Chopta	CH	79.20422º E	30.48255º N	2700	12
Population 7	Dugalbitta	DG	79.17542º E	30.48389º N	2500	12
Population 8	Baniyakund	BK	79.18036º E	30.48207º N	2450	12
Population 9	Garwali	GW	79.10631º E	30.48624º N	2300	12



A

B

C

D

Fig 3.2: Plant material collected from different geographical locations

(A) Garwali, (B)Pothivasa, (C)Dugalbitta, (D)Tungnath

3.1.5 Laboratory instruments, Plastic wares, Glass wares

In the present study, all the chemicals utilized were of analytical grade and were obtained from reputable suppliers such as Merck and Himedia Pvt Ltd in India. The Glass wares, plastic wares, Eppendorf tubes, micropipettes used were procured from standard companies like tarsons, borosil, blue star etc.

The various instruments used in the present study were from standard companies. Electrophoresis Apparatus Vertical: Mini, Midi & Maxi from Genei, Centrifuges and Micro-centrifuges from Remi, India, UV Spectrophotometer Shimadzu, Japan, Spectrophotometer Single Beam and Dual Beam Systronic, India, Thermal Cycler gradient (Thermo Fisher Scientific), UV Gel Documentation system (Analytic Jena), Deep Freezers (-20^0C & -80^0C).

3.2 Methods

3.2.1 Sterilization of Glass wares and Plastic wares

In order to avoid and control contamination the Glass wares and plastic wares were washed properly in tween 20, followed by tap water and autoclaved distilled water. The glass wares were then sterilized in Hot air oven to make them contamination free. Chemical sterilization is sometimes required that involves using a chemical agent to kill microorganisms. Glassware and plastic ware are soaked in a sterilizing solution such as hydrogen peroxide, formaldehyde, or ethylene oxide. This method is effective for sterilizing heat-sensitive items. Small items like Glass slides, pipettes, PCR tubes, Eppendorf's, tips etc. were sterilized by UV sterilization which is a non-thermal method that uses ultraviolet light to kill microorganisms. The items to be used in DNA isolation were exposed to UV light for a specified period before use to make them contamination free.

3.2.2 Phytochemical Analysis for Active Component

Phytochemical analysis is a process of identifying, isolating, and characterizing the bioactive compounds found in plants. The term phytochemicals or secondary metabolites"is commonly used to describe these compounds, which encompass a diverse array of chemical classes such as alkaloids, flavonoids, terpenoids, and phenolic compounds. The leaf samples collected from

different three different sampling sites were kept separately in paper bags till further use. There were total 7 populations which were analysed for the presence of active component.

3.2.2.1 Preparation of Plant Extract

The leaves were cleaned with water, sterilized, and washed with distilled water before being dried in the shade. The plant material was dried at a temperature of 30 ± 2 °C. The leaves were crushed into a powder using a crusher and pestle after drying. For a uniform and fine particle size, the resultant powder was subsequently run through a sieve with a mesh size of 0.3 mm. 20 gm each of fine powder of each population from different sampling site was then subjected to extraction in 200 ml of solvent using Soxhlet Apparatus. Different solvents used are Chloroform, Acetone, Methanol and Distilled Water (Table 3.2). These extracts were concentrated to obtain solid mass which was further dissolved in dimethyl sulfoxide to obtain the desired concentration. The extract was then stored at 4 ^{0}C till further analysis.

Table 3.2: Physical properties of common solvents used in bioactivity and Phytochemical analysis.

Solvent	**Polarity Index**	**Boiling point**	**Specific Gravity (20^{o}C)**
Chloroform	4.1	61	1.486
Acetone	5.1	56	0.791
Methanol	5.1	65	0.792
Water	9.0	100	-

3.2.2.2 Qualitative Phytochemical Analysis

The extracts were re-dissolved in dimethyl sulfoxide to make stock solution (10mg/ml). The solvent extracts of *Rheum* leaves underwent qualitative chemical tests to identify various plant components, including alkaloids, carbohydrates, proteins, sterols, glycosides, tannins, saponins, amino acids, and other phyto-constituents. These constituents are known to be accountable for the pharmacological and biological activities of the plant. Preliminary chemical tests were conducted

following standard protocols described in several previous studies, including Thakre et al., (2021), Trease and Evans (1989), Sofowora (1993), and Herborne (1973). [142,143,144,145]

The extracts were then exposed to different chemical tests to detect the presence or absence of specific components present in it. The methodology followed has been described as follows:

Detection of Carbohydrates

Each crude extract (CE) was separately diluted in 4ml of distilled water before being filtered. The filtrate that resulted was examined using Molish's Test, Fehling's Test, Iodine Test, and Benedict's Test.

a) **Molisch's Test**: Small amount of CE mixed was with 2 ml of Molisch reagent, shaken properly and 2 ml of conc. H_2SO_4 was carefully added along the sides. The presence of Purple ring at interphase confirms the presence of carbohydrates in the extract.
b) **Benedict's Test**: 5 ml of benedict reagent added with small amount of Crude extract and then boiled properly to obtain reddish brown precipitate (Ppt) confirming the occurrence of carbohydrates.
c) **Iodine Test**: The CE was combined with iodine solution (2 ml) to obtain Dark Blue or purple color indicating the presence of carbohydrates and glycosides.
d) **Fehling Test**: Fehling A and Fehling B (equal volume) was added to 2 ml of CE, mixture was boiled to obtain Brick red precipitate showing the occurrence of carbohydrates.

Detection of Proteins

a) **Ninhydrin Test**: To CE, 2 ml of 0.2 % Ninhydrin Reagent was added, mixed well and bring to boil to obtain violet color indicating the presence of proteins and amino acids in the test sample.
b) **Biuret Test**: To 1ml of 40 % NaOH 2 ml of CE was added, mixed properly. The presence of violet color indicated the presence of proteins.
c) **Xanthoproteic Test**: To CE 20 % NaOH added along sides carefully. Orange color precipitates indicates the presence of proteins in the sample.

Detection of Glycosides

a) **Liebermann's Test**: To chloroform and acetic acid (2ml), CE was added and after cooling the mixture conc. H_2SO_4 was added along the sides. The existence of the glycone part of the glycoside, or the steroidal nucleus, was indicated by a shift in color from violet to green.
b) **Salkoswki's Test**: In a test tube CE, chloroform, H_2SO_4 (2 ml) was added and mixture was shaken slightly to obtain a reddish brown color precipitate indicating the presence glycoside.
c) **Keller Kilani Test**: CE combined with 2 ml acetic acid and 1-2 drops of 2 % $FeCl_3$ and added to a separate test tube having 2 ml of concentrated H_2SO_4. The presence of cardiac glycosides was shown by the appearance of brown ring at the interphase.

Detection of Saponins

Foam Test or Frothing test: 0.5 ml of CE was mixed with 5 ml of distilled water in a test tube and was shaken vigorously. The formation of stable froth indicates the presence of saponins.

Detection of Phenols and Tannins

To 2 ml of 2 % solution of Ferric Chloride, 2 ml of CE was added. A blue green/ or black colour indicates the presence of phenols and tannins.

Detection of Flavonoids

Alkaline reagent test: In a test tube crude extract was mixed with 2 ml of 2 % NaOH solution. An intense yellow colour turned colourless on addition of few drops of diluted acid indicating the presence of flavonoids.

Detection of Steroids

To CE, 2 ml of chloroform along with conc. H_2SO_4 was added along the sides. The appearance of red colour in the chloroform layer indicated the presence of steroids.

Detection of Terpenoids

Crude extract was dissolved in 2 ml of chloroform and solution so formed was evaporated till dry. To this solution, 2 ml of H_2SO_4 was added and heated for 2 minutes. Greyish colour indicated the presence of terpenoids.

Detection of Alkaloids

In a test tube CE was mixed with 2ml Hager's reagent i.e. saturated picric acid. The presence of alkaloids was indicated by the appearance of yellow colour in the test tube.

Detection of Fixed oils in crude sample

Two layers of Whatman paper were sandwiched together with two drops of the extract. The presence of fixed oils is indicated by the emergence of oil strain on the filter paper.

3.2.2.3 Quantitative Phytochemical Analysis

a) Determination of Total Phenolic Content

The total phenolic component was determined using standard curve. A stock solution of 0.5 mg/ml of emodin dissolved in methanol was taken as standard. A dilution series of 0.04, 0.08, 0.12, 0.16, 0.20 mg/ml was prepared in methanol using the stock standard. Methanol was taken as blank for measuring the optical density of samples. The UV-Visible Spectrophotometer was used to determine the absorbance at 437 nm. The calibration line was used to determine the concentration of emodin in the sample, and the amount of phenolic compounds was expressed as emodin equivalents (mg of emodin/g of extract). [146,147,148]

b) Thin layer Chromatography

Thin layer chromatography can separate complex mixtures of compounds based on their different physical and chemical properties, such as polarity and solubility. This technique is especially useful in separating and analyzing natural products, pharmaceuticals, and other complex mixtures. To determine the 'presence or absence' of emodin in the samples, TLC was performed. Chromatography was performed on 7×4 inches pre-coated Silica gel coated TLC plates without

florescence indicator (Merck, Germany). The samples of the standard solution Standard 1 (0.2 g/ml Emodin dissolved in methanol) and Standard 2 (0.5 mg/ml Emodin dissolved in methanol) and extracts from *Rheum* leaves were spotted on the TLC plate with the help of capillary and allowed to dry. Chromatograms were created in a horizontal glass chamber with the adsorbent layer facing down using solvent mixes of hexane, chloroform, and acetic acid in the ratio of (6:1:0.1). Iodine vapors were used to view 15 areas until brown spots appeared on the TLC sheet. To gather calibration data, the experiments were conducted three times.

Rf (retention factor) value is a measure of the distance a compound travels on a TLC plate relative to the distance the solvent front travels. It is an important parameter used in TLC to identify and characterize compounds in a mixture. The distance that the compound travels from its starting point to the spot's center is divided by the distance that the solvent front travels from its starting point to the spot's center to arrive at the Rf value. The Rf value is denoted mathematically as follows:

Rf = distance traveled by the compound / distance traveled by the solvent front

The Rf is a scale-less or non-dimensional quantity and can range from 0 to 1. The higher value of Rf indicates that the compound under study is more soluble in the mobile phase and it will travel faster on the TLC plate. Conversely, compounds that are less soluble in the mobile phase will have lower Rf values and will travel slower on the TLC plate.

3.2.3 Antimicrobial Activity of Plant Extracts

3.2.3.1 Test organism

Five bacterial and two fungal strains were used in the present study. Two strains (*Staphylococcus aureus (MTCC 96), Bacillus subtilis (MTCC 441)*), were Gram positive and three strain (*Escherichia coli (MTCC 739), Pseudomonas, Salmonella*) were gram-negative. Fungal strains were *Candida (MTCC 183)* and *Aspergillus (MTCC 281)*. All the bacterial and fungal strains were procured from laboratory of Himalayan School of Biosciences, Swami Rama Himalayan University, Jollygrant, Dehradun, Uttarakhand.

3.2.3.2 Preparation of inoculum

For preservation, stock cultures were stored on nutrient agar slopes at 4 °C. A loopful of cells from the stock cultures were transferred to test tubes containing Mueller-Hinton broth (for bacteria) or Potato Dextrose Agar (for fungi) to create active cultures for tests. Following that, the cultures were left undisturbed for 24 hours at 37 °C and 48 hours at 27 °C, respectively. Then, 0.2 ml of the culture was injected and allowed to incubate until it attained a turbidity of equal to the standard 0.5 McFarland solution, measured at 600 nm, which is equal to a bacterial concentration of 106-108 CFU/ml.

3.2.3.3 Screening of Antimicrobial assay

The evaluation of the antibacterial activity of plant extracts was done using the disc diffusion method, as described by Baur *et al*. in 1966,[149] using Mueller Hinton Agar (MHA), Himedia. The in vitro antimicrobial activity screening was carried out by pouring 15 ml of molten media into sterile petri plates to create MHA plates, which were then given 5 minutes to harden. The plates were evenly swabbed with a 0.1% inoculum suspension, and the inoculum was allowed to dry for 5 minutes. Using a sterile cork borer, 6 mm-diameter holes were made, and 50 µL of each plant's CE were placed into each well. In order to ensure proper diffusion, the plates were left on the bench for an hour.

Each extract's mother solvent was utilized as a control. A clear ruler was used to measure the inhibition zones that had formed around the discs after the incubation time (in mm).

3.2.3.4 Statistical analysis

All the experiments were conducted in triplicates. The results were presented as the mean value ± standard deviation (SD). The level of significance was determined using one-way ANOVA using a statistical package program (SPSS 10.0).

3.2.4 Standardization of protocol for Isolation of Genomic DNA

3.2.4.1 Selection of plant material for DNA Extraction

Selecting the appropriate plant material for DNA extraction is essential to obtain fine quality of DNA. The age of the plant can affect DNA yield therefore for the extraction of genomic DNA

younger plant leaves were selected as they contain more high-quality DNA than older leaves. It's important to ensure that the plant material is healthy and free from any physical or chemical damage.

3.2.4.2 Genomic DNA Extraction

Young juvenile leaves of Rheum plant were selected for the isolation of total genomic DNA by a modification in CTAB method given by Doyle and Doyle (1990).[150] The extraction was carried in presence of different detergents and varied molar concentration of components of extraction buffer to eliminate or minimize the secondary metabolite and protein contamination. The buffer components and the conditions were standardized that yielded a good quantity and protein free genomic DNA. This protocol after standardization will be further used for the isolation of genomic DNA from various sources of the plant under study.

3.2.4.2.1 Solutions and Reagents

- CTAB Buffer (Cetyl-trimethylammonium Bromide): (Preheated at 65°C)100 mM Tris-HCl (pH-8.0), 20 mM EDTA (Ethylenediaminetetraacetic acid) (pH-8.0), 1.5 mM NaCl, 2% CTAB, 2% polyvinylpyrollidone (PVP), 5 mM Ascorbic acid and 0.2% β-mercaptoethanol.
- 5 M Amonium acetate solution
- Chloroform: Isoamyl alcohol (24:1)
- Ethanol 76% ,96 %
- Chilled Isopropanol
- TE Buffer: (Tris HCl 10mM, EDTA 1mM) pH 8.0
- Wash buffer: (998µl of 96%ethanol and 2µl of ammonium acetate)
- RNAse (10mg/ml)

3.2.4.2.2 DNA Extraction Protocol (Fig 3.3) [151,152,153]

i. The extraction buffer was made freshly by adding all the components and preheated in water bath at 65ºC. β- mercaptoethanol was added to the extraction buffer instantly before use.

ii. Wash the leaf material properly with tap water, distilled water then with 70 % ethanol followed by distilled water again to get rid of any impurities. If the plant sample is collected from any microbial affected place, the leaves should be washed additionally with tween 20.

iii. 0.5 gm of leaf sample were grinded to fine paste using pre cooled mortar and pestle with 1 ml of preheated CTAB extraction buffer. Swirl gently with the help of pestle to make a slurry like consistency. The content was transferred to microcentrifuge 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 so formed was vortexed carefully for few seconds and incubated (60° C) preferably in a water bath for 30 min with intermittent mixing after every 10 minutes. Bring the sample to room temperature before proceeding to the next step.

v. Mix an equal volume of Chloroform: Isoamayl alcohol (24:1) followed by inversion mixing of Eppendorf.

vi. The solution so obtained was then centrifuged at 15,000 rpm for 10 minutes at RT.

vii. With the help of a wide bore pipet, 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).*

The extraction steps (v-vii) can be repeated, if necessary to obtain a clear aqueous phase.

viii. The solution was centrifuged at 10,000 rpm at 4° C for 10 minutes to obtain pellet. Carefully the supernatant was discarded without disturbing the pellet and wash the pellet with 998µl of 76% ethanol and 2 µl of 10mM ammonium acetate for at least 30 minutes. *(this ensures the removal of any residual salts and increases the purity of DNA)*

ix. The washing was done by centrifuging the solution 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. Let the pellet air dry or vacuum dry at room temperature. (*Ensure the proper removal of ethanol, as it can hinder the amplification of DNA during PCR reaction).*

xi. Add 100 µl of TE Buffer followed by 3 µl of RNAse (10mg/ml) and kept undisturbed at RT for 30 minutes. The DNA was extracted again with Chloroform: IAA followed by precipitation with isopropanol. *(Addition of RNAse is optional)*

xii. Spool out the DNA, vacuum or air dry, add 200 µl of TE Buffer and stored at -20° C and allowed to suspend properly.

xiii. Stored at-20° C till further use.

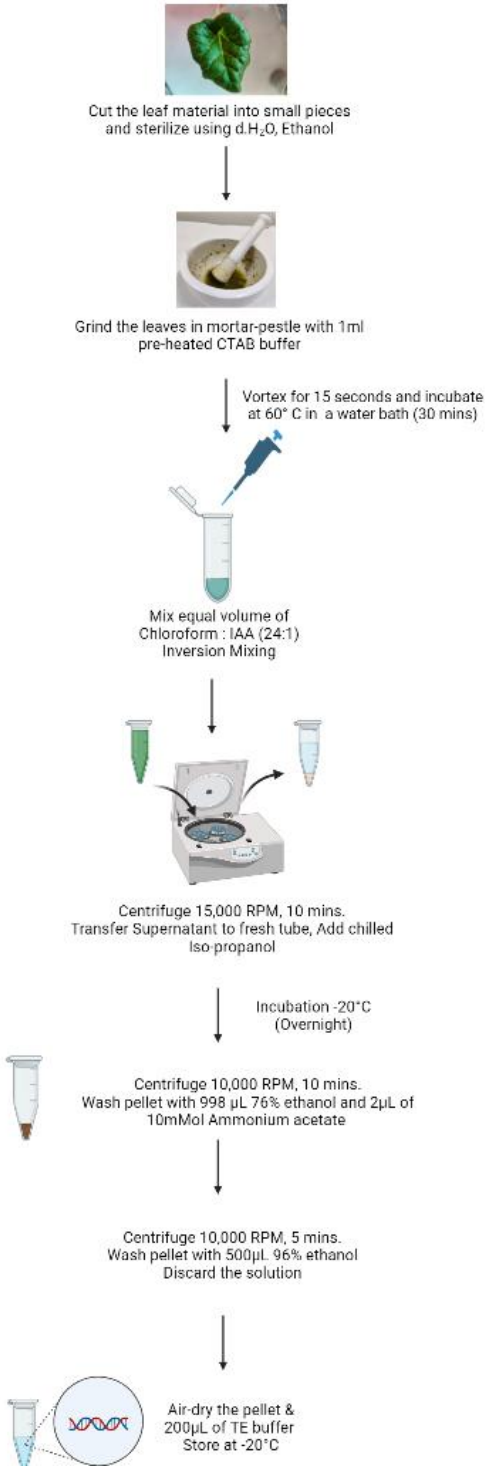


Fig 3.3: **Pictorial representation of modified CTAB DNA isolation Protocol**

3.2.4.3 Quantification of Isolated DNA

The quantification of genomic DNA was done manually, using Shimadzu Spectrophotometer UV-1800. The yield and purity was determined by measuring the absorbance at $A_{(260/280)}$ nm. The levels of isolated DNA purity were evaluated by the calculating the ratio of absorbance at 260 nm and 280 nm. Firstly, the absorbance was set at zero by using 3 ml TE buffer in a cuvette as blank. Then dilution of sample (30 µl of sample and 2970 µl of diluent) was used to measure the optical density at 260 and 280nm. The absorbance ratios in the range of 1.7- 2.0 are generally assumed to be the good quality genomic DNA that can be used further for amplification using PCR. All the extracted DNA samples were further diluted according to need of further experimentation. [154,151] The quantification analysis was carried out separately for all the accessions.

The DNA concentration is estimated as

Concentration (µg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50µg/ml

where

A_{260} is the maximum absorbance of DNA sample

A_{320} is the measurement of turbidity in the sample

Conversion Factor A_{260} of 1.0 = 50µg/ml pure dsDNA

Total yield is calculated by multiplying the DNA conc. by the final total purified sample volume.

DNA yield (µg) = DNA concentration × total sample volume (ml)

3.2.4.4 Qualitative Analysis of Isolated DNA

The isolated DNA samples were further examined by running the genomic DNA on 0.8% Agarose with 3 µl of ethidium bromide (0.25 µg/ml) prepared in 1 X buffer for the qualitative examination of extracted DNA. [154,151] A DNA Marker (HI Media) was run on each gel to determine the molecular weight of isolated DNA by comparing them with the DNA ladder or marker. The gel was run at a continuous voltage of 5 V/cm and allowed to run till the bands reached ¾ length of the gel. The gel was visualized in GelDock (Analytic Jena) and photographed under UV light using

a digital camera. The absence of smearing or degraded DNA bands and the presence sharp band at the expected size indicates high-quality DNA.

3.2.4.5 Dilution Preparation

The dilutions are often prepared to ensure that the conc. of DNA in a sample is within the range required for downstream applications, such as PCR, sequencing, genetic analysis. The concentration of DNA used in the present study is 20 ng/µl. All the DNA samples needs to be bring down to the same concentration of DNA before proceeding for PCR analysis. Dilutions can be obtained using the formula:

$$\mathbf{C_1V_1 = C_2V_2}$$

where,

C_1 = Initial conc. of stock of total genomic DNA

C_2 = Conc. of DNA sample required for analysis

V_1 = Volume of DNA required to be added in dilution

V_2 = Volume of final sample

The appropriate volumes of DNA sample and diluent were pipetted out into a clean tube and mixed thoroughly by pipetting in and out several times. The diluent used in the present study is autoclaved DNase and RNase free water. The diluted sample is properly labelled with the dilution factor, the concentration of the original sample, and any other relevant information. The stock solution was stored at -20ºC till future use.

3.2.5 Molecular Marker Analysis

3.2.5.1 Screening of RAPD Primers

i. Aliquots Preparation

Aliquot preparation is the process of dividing a sample or a solution into smaller, equal volumes. Aliquots are typically prepared by measuring out the appropriate volume of sample or solution and then transferring it to a clean, sterile container. Preparing aliquots of PCR reagents is often used to minimize variability in PCR reactions and to ensure that there are enough reagents for multiple

reactions. PCR reagents, including the DNA template, primers, dNTPs, buffer, and Taq polymerase, are prior thawed. All the components are available in ready to use form apart from the primers. The deionized water according to the Performa needs to be added to the primer mixes to make a final solution. After adding the deionized water, the primer solution was allowed to rest for 15 minutes ensuring the proper mixing of the components. The working primer concentration i.e.0.2 µM was then prepared using the above prepared stock solution of primer mixes. The aliquot of master mix was then prepared by calculating the volume of master mix required for each individual reaction, depending upon the total volume of each reaction and the concentration of each reagent in the master mix. All the PCR aliquots were stored at the appropriate temperature, typically -20°C or -80°C, until ready to use.

ii. Standardization of PCR amplification Conditions

Various components of Polymerase Chain Reaction like the concentration of template DNA, Mg^{++} ions, primer sets, Taq DNA polymerase, dNTP's were optimized for getting the maximum resolution of the amplified loci in case of RAPD for the plant under study. The concentration of one component was optimized at a time b keeping the concentration of other component at constant.

Optimization of Genomic DNA Concentration

The concentration of target sequence in the PCR reaction is generally in nanograms (15-45 ng), therefore the optimized concentration of each DNA sample in 1µl aliquot was used directly. The optimal conc. was found to be 15- 20 ng/µl for RAPD. Absence of amplification with lower concentration and presence of smear or non-specific amplification was observed at higher concentration of genomic DNA.

Optimization of Primer Concentration

Primer concentration that gave intense band and specific amplification of prominent reproducible loci was observed at concentration of 0.2µM for the PCR reaction. Concentrations below and above 0.2µM gave faint band resulting in the less yields of the amplified product and smeared visualization. Thus 0.2µM concentration was used while optimization the further components. Higher primer concentration may promote non-specific product formation and may increase

generation of primer artifacts by non-specific annealing or complementation in the primer. However, primer concentration may allow complementary strand annealing to compete with primer annealing, reducing product yield.

Optimization of *Taq* DNA Polymerase

A concentrated stock of *Taq* DNA polymerase was used, having 5 units of enzyme per 1µl, 0.2µl of this mixture corresponding to 1U per reaction mixture was optimized for excellent results. The concentration more or less than 0.2µl of enzyme resulted in smear and hazy background.

Optimization of dNTPs and Mg^{++} Ions

DNA synthesis requires free (dNTPs). The four dNTPs - dATP, dGTP, dCTP, and dTTP - are used at equimolar concentrations to minimize errors during the process. However, it is important to avoid a final concentration of more than 50mM of total dNTPs in the PCR, as it can hinder Taq DNA polymerase activity. The 2.5 mM concentration of each dNTP was found to be the optimum concentration for maximum amplification in all the RAPD analysis that resulted in highest number of amplified products.

The optimized Mg^{++} concentration was observed at 3 mM in the RAPD analysis that was needed to maintain the fidelity of *Taq* DNA polymerase. The reduced concentration Mg^{++} resulted in non-specific amplification or no amplification of PCR product, while excess of Mg^{++} ions resulted in the formation of smear resulting in non-specific amplified product.

Optimization of Annealing Temperature

Efficient annealing of oligonucleotide primer to the target sequences of separated DNA strands was found in the range of 30^0C- 37 ^{0}C for RAPD depending upon the primers used to carry on the PCR cycle. After running repeated cycles of temperature variations gradient for different primers the temperature resulting in the maximum number of PCR amplified products was selected. Meanwhile any alteration in the annealing temperature resulted in the low reproducibility of the amplified product.

Optimization of Number of Cycles

By using optimized condition for all other factors, number of cycles was standardizing at 40. Best results yield of intensely amplified product were obtained at 40 numbers of cycles.

The table (Table 3.3) below shows the total number of primers, the selected primers, their sequences, and some characteristics of amplification products in the analysed accessions:

Sr no	**Coding**	**Oligo Name**	**Tm (ºC)**	**Experimental Tm (ºC)**	**Sequence (5`- 3`)**	**Maximum No. of Bands**
1	RD2	**OPA 09**	27	37	GGGTAACGCC	14
2	RD3	**OPB 09**	27	37	TGGGGGACTC	12
3	RD4	**OPE 06**	25	37	AAGACCCCTC	12
4	RD5	**OPL 10**	25	31	TGGGAGATGG	09
5	RD6	**OPL 19**	25	37	GAGTGGTGAC	13
6	RD7	**OPP 11**	27	30	AACGCGTCGG	09
7	RD8	**OPP 12**	25	37	AAGGGCGAGT	12
8	RD9	**OPA 17**	25	27	GACCGCTTGT	11
9	RD10	**OPA 19**	25	37	CAAACGTCGG	13
10	RD11	**OPB 04**	25	37	GGACTGGAGT	14
11	RD12	**OPE 08**	25	31	TCACACGGT	10
12	RD13	**OPD 04**	25	35	TCTGGTGAGG	13
13	RD1	**OPP 13**	23	Experimental Melting temperature could not be standardized	AAAGTGCCTC	No Amplification
14	RD 14	**OPD 09**	25		CTCTGGAGAC	
15	RD 15	**OPP 05**	25		AGGGGTCTTG	

Table 3.3: RAPD Primers utilized for *Rheum species*

iii. RAPD PCR Analysis

The Random Amplified Polymorphic DNA analysis was initially carried out with 15 primers[133] (*Persson et al., 2000*) out of which only 12 were selected to carry out RAPD PCR for the determination of GD of *Rheum* species. The primers were obtained from Sigma Aldrich The PCR

amplification was conducted in a 15µl reaction mixture using a thermal cycler (Applied Biosystems by Thermo Fisher Scientific). The PCR products were run on 1 % agarose gel and bands were observed in Gel documentation system photographed with a digital camera.

After optimization of all the reaction components following reaction mixture was considered to be the standardized for RAPD marker. The components of reaction components are as follows:

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

iv. Cycling Parameters

The reaction mixture was given a brief spin and aliquoted into 0.2 ml thin walled clear PCR tubes and then thermocycler was allowed to run. The PCR amplification cycle consisted of Denaturation at 95°C for 5 min, repeat for 40 cycles: (Denaturation at 94°C for 1 min, annealing at 37°C for 1 min, Extension at 72°C for 1 min), Final extension at 72°C for 8 min, hold at 4°C till stored for further use. The annealing temperature varied depending upon the primer used in every cycle.

3.2.5.2 Screening of ISSR Primers

i. Aliquots Preparation

Aliquots are typically prepared by measuring out the appropriate volume of PCR master mix solution and then transferring it to a clean, sterile container. The volume of the aliquot depends on the amount of sample or solution available and the requirements of the protocol. For the preparation of master mix always clean and sterile equipment are used to avoid cross-

contamination between samples when preparing aliquots. Similarly, as discussed earlier except from primers all other components are available in ready to use form. Prescribed amount of autoclaved de-ionized water was added to the commercially available primer samples. The primers were allowed to settle for 15 minutes to ensure proper diffusion of all the components. The working primer concentration for ISSR PCR 0.4µM was then prepared using this stock solution. Finally, all the components were then stored at the appropriate temperature i.e. at -20ºC to prevent damage and degradation.

ii. Standardization of PCR amplification Conditions

As described earlier various components of Polymerase Chain Reaction like the concentration of template DNA, Mg^{++} ions, primer sets, Taq DNA polymerase, dNTP's were optimized for getting the maximum amplification for ISSR PCR.

The optimum concentration of Genomic DNA was found to be 15- 20 ng/µl for ISSR primers for the determination of genetic variations in *Rheum* species. The primer concentration that gave intense band and specific amplification of prominent reproducible loci was observed at concentration of 0.4µM for the ISSR PCR reaction. A concentrated stock of *Taq* DNA polymerase was used, having 5 units of enzyme per 1µl, 0.15µl of this mixture corresponding to 1U per reaction mixture was optimized for excellent results.

The 2.5 mM concentration of each dNTP was found to be the optimum concentration for maximum amplification in all the ISSR analysis that resulted in highest number of amplified products. The optimized Mg^{++} concentration was observed at 2 mM in the ISSR analysis that was needed to maintain the fidelity of *Taq* DNA polymerase.

The optimization of annealing temperature is the crucial step in ISSR analysis. After several repeated cycles of gradient PCR for different primers the temperature resulting in the maximum number of PCR amplified products was selected. The annealing temperature vary according to the primer used. Meanwhile any alteration in the annealing temperature resulted in the low reproducibility of the amplified product.

By using optimized condition for all other factors, number of cycles was standardizing at 40. Best results yield of intensely amplified product were obtained at 40 numbers of cycles. The table (Table

3.4) below presents the total number of primers, the chosen primers, their sequences, and various characteristics of the amplification products observed in the analysed accessions.

Table 3.4: ISSR Primers utilized for genetic diversity assessment of *Rheum species*

Sr no	Coding	Oligo Name	Tm (ºC)	Experimental Tm (ºC)	Sequence (5`-3`)	Maximum No. of Bands
1	IR 6	UBC 834	51.1	52.7	AGAGAGAGAGAGAGAGCTT	14
2	IR 7	UBC 835	51.5	52.7	AGAGAGAGAGAGAGAGCTC	13
3	IR 9	UBC 842	49.7	52.7	GAGAGAGAGAGAGAGACTG	11
4	IR 10	UBC 811	43.3	43.3	GAGAGAGAGAGAGAGAC	11
5	IR 12	UBC 808	46.8	47.1	AGAGAGAGAGAGAGAGC	20
6	IR 13	UBC 809	46.6	48.7	AGAGAGAGAGAGAGAGG	15
7	IR 14	UBC 868	47.8	48.7	GAAGAAGAAGAAGAAGAA	16
8	IR 15	UBC 840	48.2	47.1	GAGAGAGAGAGAGAGACTT	15
9	IR 16	UBC 841	58.1	60.0	GAGAGAGAGAGAGAGACGACTC	15
10	IR 17	UBC 889	59.2	59.2	AGTCGTAGTACACACACACACAC	18
11	IR 18	UBC 891	62.7	64.0	AGTACGAGTTGTGTGTGTGTG	12
12	IR 19	UBC 890	65.6	67.0	ACGAGTAGGGTGTGTGTGTGTGT	13
13	IR 20	UBC 888	67.6	67.0	CTGAGTCGTCACACACACACACA	16
14	IR 1	UBC 825	49.3	Experimental Melting temperature could not be standardized	ACACACACACACACACT	No Amplification
15	IR 2	UBC 836	49.3		AGAGAGAGAGAGAGAGCTA	
16	IR 3	UBC 844	49.3		CTCTCTCTCTCTCTCTAGC	
17	IR 4	UBC 816	43.3		CACACACACACACACAT	
18	IR 5	UBC 855	49.7		ACACACACACACACACCTT	
19	IR 8	UBC 817	52.8		CACACACACACACACAA	
20	IR 11	UBC 807	42.5		AGAGAGAGAGAGAGAGT	

iii. ISSR -PCR Analysis

For ISSR-PCR analysis 20 primers (Table 3.4) obtained from Sigma Aldrich were used *(Wang et al., 2012)* were used initially.[29] Out of which only 13 with maximum number of amplified bands were selected for additional analysis. PCR amplification was performed in a 20µl reaction mixture using a thermal cycler. The resulting PCR products were visualized on a 1% agarose gel, and the bands were observed and recorded using a Gel documentation system with a digital camera under UV light. The components of the reaction mixture are as follows:

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

iv. Cycling Parameters

The amplification cycle comprised the following steps: one cycle of denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at a specific temperature for each primer for 30 seconds, and extension at 72°C for 1 minute. After the amplification cycles, a final extension step was conducted at 72°C for 10 minutes. The reaction mixture was then held at 4°C until taken for storage.

The PCR products obtained were subsequently visualized using agarose gel electrophoresis.

3.2.6 Gel Electrophoresis

An essential method for separating and evaluating DNA fragments according to their size is agarose gel electrophoresis. By comparing the dimensions of unknown DNA fragments to parallel

measurements of known molecular weight markers, it provides a reliable and precise approach for measuring the size of DNA fragments.[155] Apart from this Agarose gel electrophoresis is a quick and simple way to check the purity of a DNA sample. For example, if a DNA sample contains contaminants such as RNA or protein, they may show up as additional bands on the gel. In molecular marker analysis it is an important quality control step as it is used to confirm that PCR amplification was successful or not.

i. Preparation of Gel

- Usually for PCR product analysis 1 % and for genomic DNA visualization 0.8% agarose gel are used. The agarose gels are casted according to w/v % solution i.e. for 0.8 % gel, 0.8 gm of agarose powder in 100 ml of TAE or TBE is required. The conc. of agarose in the gel is also dependent on the size of DNA fragment to be separated. In an Erlenmeyer flask, 1 gm of agarose (Genei, Himedia) is added and to it 100 ml of TAE (1X) buffer which is also known as running buffer or electrophoretic buffer is added.
- Mix by swirling and heat up the mixture to completely dissolve the components. The volume of agarose gel to be made depends upon he sizes of casting tray used. Boil the mixture till the solution becomes transparent.
- Allow the agarose to cool down to 65 °C or to a temperature that your hand can tolerate. Now add Ethidium bromide (EtBr) 0.5 μg/ml concentration. EtBr is a carcinogen, so proper care should be taken while handling it. Give the components a nice swirl before pouring in the casting tray.
- Place the gel tray into the casting apparatus and set the comb in the desired position. Carefully pour the molten agarose solution into the casting tray, ensuring that there are no air bubbles. Allow the agarose to solidify at room temperature. Once the agarose gel has set, carefully remove the comb from the gel. Insert the gel cassette, containing the solidified agarose gel, inside the electrophoretic apparatus.

  The gel is now ready for use in electrophoresis experiments.

ii. Loading and Separation of DNA Fragments

- Before loading ensure that the enough amount of electrophoretic buffer i.e. 1 X TAE is present in the electrophoretic tank. The gel should be submerged in the buffer.

- Pipette out 2 µl of Loading dye 6X conc. '(comprising of 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol)' commercially available from HIMedia on a parafilm for each sample. For loading the DNA sample into the gel, 2µl of loading dye with 8 µl of DNA sample is used. This ratio can vary subject to the nature and conc. of DNA present in the sample. Loading dye help to track the movement of DNA and it also let the sample to descend into the gel.
- Cautiously mix the loading dye with sample with the help of micropipette. Load the samples in the well carefully. Appropriate ladder or size marker should also be loaded in one well with the DNA samples.
- Switch on the power supply and set the desired voltage. Initially at 50 V than at 80-120 V depending upon the size of the gel.
- Allow the gel to run until the dye or sample bands have migrated to an appropriate distance, typically about two-thirds of the gel length. Observe the gel under appropriate lighting conditions to visualize the separated bands and patterns of the samples.

iii. Observing Separated DNA fragments

- Carefully with gloves on, remove the gel from the electrophoretic tank, drain excess of buffer and place the gel inside the gel documentation system.
- Turn on the UV light and observe the bands. The DNA bands should be visible as orange fluorescent bands. Capture the image using a digital camera attached to the gel documentation system.
- Dispose of the gel properly by keeping it in bleaching powder for two days and then discarding it. The images obtained will be used for further analysis for the evaluation of genetic variations in *Rheum* species.

3.2.7 Data Analysis

For the analysis of genetic diversity only consistent, bright and reproducible bands were scored manually as binary data indicated by 1 (presence) and 0 (absence) obtained from RAPD and ISSR analysis. The smeared and weak bands were excluded from coding. This will generate a binary matrix of 1s and 0s representing the DNA profile of each sample. By calculating a number of parameters, including the average number of alleles per locus (na), the effective number of alleles per locus (ne), the expected heterozygosity (Ht), the observed heterozygosity (H_0), and the

Shannon Information index (I), the binary matrices of RAPD data were prepared and used to assess genetic variability. Additionally, POPGENE version 1.32 was used to calculate Nei's gene diversity (1973).

The variance among the populations was estimated using the Wright fixation index (F_{st}), and its statistical significance was evaluated using 1000 permutations of the genotypes among the populations. The formula (Ht - Hs) / Ht was used to determine the total genetic diversity (Ht), total genetic diversity within populations (Hs), and Nei's coefficient of genetic differentiation between populations (G_{st}) at the species level.

The formula $Nm = 0.5 (1 - G_{ST}) / G_{ST}$ was used to calculate the gene flow (Nm) across populations, which represents the average number of migrants exchanged per generation.[156] Populations are thought to be in Hardy-Weinberg equilibrium for all calculations. Additionally, Nei's unbiased genetic distance and genetic identity for all pairwise combinations of populations were used to investigate the genetic links among populations. Using POPGENE version 1.32, a UPGMA dendrogram was created based on the matrix of Nei's unbiased genetic distance.

In addition to this, polymorphic information content (PIC) which is defined as the value of marker for detecting polymorphism among the populations was also calculated. To calculate the Polymorphic Information Content (PIC), the formula PICi = 2fi (1 - fi) was used, where fi represents the frequency of the amplified allele, and (1 - fi) represents the frequency of the null allele.[157] For estimating the genetic diversity among and within the population, an Analysis of Molecular Variance (AMOVA) was employed. The AMOVA was performed using Arlequin version 3.01.[158] The analysis involved grouping the molecular variance into two categories: one for inter-population variance and another for intra-population variance. The significance level of these estimates was also determined.

The data obtained from RAPD and ISSR analysis can be analyzed using various statistical methods to gain insights into genetic variation and population structure, and to inform further research and conservation efforts.