Chapter 4

RESULTS

The leaves of *Rheum* along with rhizome and few plants from different populations were procured in the month of July- September from nine different regions of Uttarakhand. The plant samples so collected from different locations were validated by Botanical Survey of India, Northern Regional Centre Dehradun, vide Accession number **1146** to validate the representativeness of the sample to ensure that the results are reliable and accurate. These samples were then further taken for analysis.

4.1 Phytochemical Analysis for active components

In present investigation seven different landraces of *Rheum emodi* from different geographical locations were used and subjected to phytochemical screening of various constituents as mentioned in Chapter 3. The coding of samples along with locations are given in Table 4.1

Table 4.1: Sampling sites for phytochemical analysis of *Rheum species*

Sample no.	Sample location	Sample Code	Longitude (E)	Latitude (N)	Altitude (Meter)
Sample 1	Garwali	GW	79.10631^{o}E	30.48624^{o}N	2300
Sample 2	Shyalmi	SM	79. 10 44° E	30. 30 24° N	2260
Sample 3	Pothivasa	PV	79.16^{o} E	30. 28° N	2000
Sample 4	Tungnath	TG	79.21699^{o} E	30.48940^{o} N	3600
Sample 5	Baniyakund	BK	79.18036^{o} E	30.48207^{o} N	2450
Sample 6	Dugalbitta	DG	79.17542^{o} E	30.48389^{o} N	2500
Sample 7	Chopta	CH	79.20422^{o} E	30.48255^{o} N	2700

4.1.1 Yield Value Determination

For the determination of yield value percentage of each extracts the dry weight of crude extract obtained after soxhlet extraction was divided by weight of dried leaf sample used, multiplied by 100. The yield of crude extract falls in the range of 2 gm to 4 gm in different leaf extracts obtained after soxhlet extraction (Table 4.2).

Table 4.2: Yield of different extracts obtained after Soxhlet Extraction

Sample Code	Solvent	Dry Weight	Volume of solvent	Crude Extract	% Yield
GW	Chloroform	20 gm	200 ml	3.4 gm	17%
	Acetone		200 ml	3.0 gm	15%
	Methanol		200 ml	2.5 gm	12.50%
	Water		200 ml	2.0 gm	10%
SM	Chloroform	20 gm	200 ml	4.0 gm	20%
	Acetone		200 ml	3.0 gm	15%
	Methanol		200 ml	3.2 gm	16%
	Water		200 ml	2.1 gm	10.50%
PV	Chloroform	20 gm	200 ml	4.6 gm	23%
	Acetone		200 ml	3.1 gm	15.50%
	Methanol		200 ml	3.8 gm	19%
	Water		200 ml	2.5 gm	12.50%
TG	Chloroform	20 gm	200 ml	3.5 gm	17.50%
	Acetone		200 ml	3.1 gm	15.50%
	Methanol		200 ml	3.2 gm	16%
	Water		200 ml	2.3 gm	11.50%
BK	Chloroform	20 gm	200 ml	3.6 gm	18%
	Acetone		200 ml	3.0 gm	15%
	Methanol		200 ml	2.8 gm	14%
	Water		200 ml	2.0 gm	10%
DG	Chloroform	20 gm	200 ml	3.8 gm	19%
	Acetone		200 ml	3.4 gm	17%
	Methanol		200 ml	3.0 gm	15%
	Water		200 ml	2.3 gm	11.50%
CH	Chloroform	20 gm	200 ml	2.8 gm	14%
	Acetone		200 ml	2.3 gm	11.50%
	Methanol		200 ml	3.1 gm	15.50%
	Water		200 ml	2.0 gm	10%

4.1.2 Qualitative Phytochemical Analysis

The results of phytochemical screening are as follows

Table 4.3: Phytochemical Screening analysis of plant extracts of *Rheum* species

Metabolite	Name of Test	Methodology	Results
Carbohydrate	Molisch's Test	C. E + mixed with 2 ml of Molisch reagent, shaken properly, 2 ml of conc H_2SO_4 along the sides	Violet ring at interphase
	Benedict's Test	C.E + 5 ml of benedict reagent and then boiled	Reddish brown ppt
	Iodine Test	C.E. + 2 ml iodine solution	Blue /Purple colour
	Fehling Test	Mix equal volume of Fehling A and Fehling B, take 2 ml of this reagent and add C.E. and boiled	Brick red ppt
Protein	Ninhydrin Test	C.E. + 2 ml of 0.2 % Ninhydrin Reagent	Violet Colour
	Biuret Test	C.E. + 1ml of 40 % NaOH, mix properly	Violet Colour
	Xanthoproteic Test	C.E. +20 % NaOH, Mix gently	Orange Colour ppt
Glycosides	Liebermann's Test	C.E. + 2 ml Chloroform + 2 ml acetic Acid, the mixture cooled in ice, and after that concentrated H_2SO_4 along the sides	Colour change from violet to green
	Salkoswki's Test	C.E. + 2 ml Chloroform + 2 ml H_2SO_4, shaken gently	Reddish brown colour ppt
	Keller Kilani Test	C.E. + 2 ml acetic acid containing 1-2 drops of 2 % $FeCl_3$. Added to fresh tube containing 2 ml of concentrated H_2SO_4	Brown ring at interphase
Saponins	Foam Test	0.5 ml of C.E. + 5 ml of distilled water, shaken vigorously	Presence of Stable froth
Phenols and Tanins	Ferric Chloride Test	C.E. + 2 ml of 2 % $FeCl_3$	Blue green or black colour
Flavonoids	Alkaline reagent test	C.E. + 2 ml of 2 % NaOH solution	Yellow colour turned colourless on addition of acid
Steroids	Steroid Test	C.E + 2 ml chloroform + concentrated H_2SO_4 along the sides	Red colour in lower chloroform layer
Terpenoids	Terpenoid Test	C.E + 2 ml chloroform, solution so formed was evaporated and then added 2 ml of H_2SO_4 and heated gently	Greyish colour
Alkaloids	Hager's test	C.E + 2ml Hager's reagent	Yellow ppt
Fixed oil	Fixed oil Test	2 drops of C.E passed between 2 filter papers	Oil strain on filter paper

Sample Code		Iodine Test	Fixed Oil	Benedict	Fehling	Molisch	Salwoski	Liebermann	Phenols	Saponins	Steroids	Alkaline Reagent	Ninhydrin	Killer Killani	Alkaloid
GW	C	+	-	-	+	+	+	+	+	-	+	-	-	+	-
	M	-	-	-	+	+	+	+	+	+	+	-	+	+	+
	A	+	-	-	+	+	+	+	+	+	+	-	-	+	+
	W	-	-	-	-	+	+	-	+	+	+	-	+	+	+
SM	C	-	+	-	+	+	+	+	-	-	+	+	-	+	-
	M	-	+	-	+	+	+	+	+	+	+	+	+	+	+
	A	-	-	-	+	+	-	+	+	-	-	-	+	+	+
	W	-	-	-	+	+	+	+	+	+	+	+	+	+	+
PV	C	+	-	-	+	-	-	+	+	-	-	+	-	-	-
	M	-	-	+	+	+	+	-	+	+	+	-	+	+	+
	A	+	-	+	+	-	-	+	+	+	-	-	-	+	+
	W	-	-	+	+	+	+	-	+	+	+	-	+	+	+
TG	C	+	-	-	+	-	-	+	+	-	-	+	-	-	-
	M	-	-	+	+	+	+	-	+	+	+	-	+	+	+
	A	-	-	+	+	+	+	+	+	+	+	-	-	+	+
	W	-	+	+	+	+	+	-	+	+	+	+	+	+	+
BK	C	+	-	-	+	-	-	+	+	-	-	+	-	-	-
	M	-	-	+	+	+	+	-	+	+	+	-	+	+	+
	A	-	-	+	+	+	-	+	+	-	-	-	-	+	+
	W	-	+	+	+	+	+	-	+	+	+	-	+	+	+
DG	C	-	+	+	+	+	+	+	-	-	-	+	+	-	-
	M	-	+	+	+	+	+	+	+	+	+	+	-	+	+
	A	-	-	-	-	+	-	+	+	-	+	-	-	+	+
	W	+	+	+	+	+	+	-	+	+	+	+	-	+	-
CH	C	+	-	+	+	-	-	+	-	-	-	-	-	+	-
	M	-	-	+	+	+	+	+	+	+	+	-	+	+	+
	A	-	+	+	+	+	+	+	+	+	+	+	-	+	+
	W	+	-	-	-	+	+	-	+	+	+	+	+	+	-

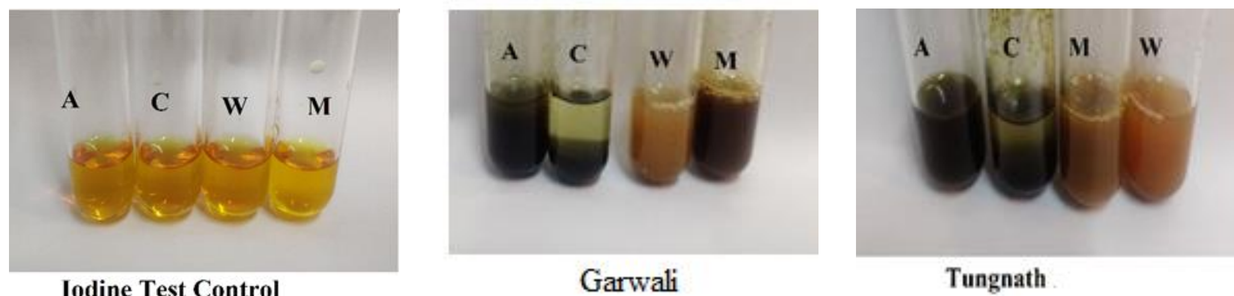


Fig 4.1: Iodine Test (Carbohydrates) in plant extracts of *Rheum* species

A= Acetone extract, C = Chloroform Extract, W = Water Extract, M = Methanol Extract

The leaves from various plant populations were extracted using different solvents (chloroform, methanol, acetone, and distilled water) following the previously mentioned method. The extracts obtained were subjected to qualitative phytochemical analysis to detect the presence of various constituents, including “alkaloids, carbohydrates, glycosides, phenolic compounds, flavonoids, amino acids, terpenoids, tannins, and saponins”. The results, summarized in Table 4.3, indicate the presence and absence of these phytoconstituents in the leaf extracts of *Rheum* species. The analysis revealed that alkaloids, saponins, proteins, carbohydrates, tannins, flavonoids, terpenes, and phenolic compounds were present in the different samples of leaf extract of *Rheum* species (Fig 4.1). Emodin and Aloe-emodin are of utmost important secondary metabolites present in the *Rheum species*, which are chemically phenols by nature. Hence the phytochemical screening confirms the presence of phenols in the extracts of all the samples of different region except for few.

4.1.3 Quantitative Phytochemical Analysis

a) Determination of Total Phenolic Content

The phenolic content was determined using standard curve. The conc. of emodin present in the sample was estimated using the calibration curve, and the content of phenolic compounds were expressed as emodin equivalent (mg of emodin/g of extract). The amount of total phenols was determined using Emodin as standard component. The absorbance for various dilutions of emodin are described in Table 4.4.

Table 4.4: Concentration vs Absorbance curve of standard Emodin

Sr No.	Concentration (mg/ml)	Absorbance (437 nm)
1	0.04	1.37
2	0.08	1.70
3	0.12	2.02
4	0.16	2.19
5	0.20	2.39

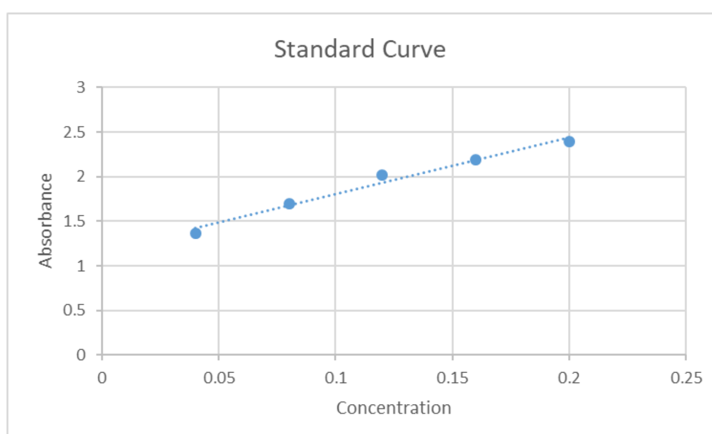


Fig 4.2: Calibration curve of standard Emodin stock

Table 4.5: Absorbance (437 nm) of samples of different populations of *Rheum*

Water	Methanol	Acetone	Chloroform
2.46	2.35	2.35	2.39
2.48	2.36	2.56	2.35
2.49	2.35	2.43	2.31
2.37	2.39	2.54	2.63
2.49	2.47	2.43	2.38
2.48	2.37	2.55	2.45
2.5	2.46	2.44	2.4

Table 4.6: Concentrations of unknown samples as depicted from standard curve

	GW	SM	PV	TG	BK	DB	CH
Methanol Abs	2.35	2.36	2.35	2.39	2.47	2.37	2.46
Conc. (mg/ml)	0.184	0.185	0.184	0.190	0.202	0.187	0.201
Acetone Abs	2.35	2.56	2.43	2.54	2.43	2.55	2.44
Conc. (mg/ml)	0.184	0.216	0.196	0.213	0.196	0.215	0.198
Chloroform Abs	2.39	2.35	2.31	2.63	2.38	2.45	2.40
Conc. (mg/ml)	0.190	0.184	0.178	0.227	0.189	0.199	0.192
Water Abs	2.46	2.48	2.49	2.37	2.49	2.48	2.50
Conc. (mg/ml)	0.201	0.204	0.206	0.187	0.206	0.204	0.207

*where **M** methanol extract, **C** Chloroform, **A** Acetone, **W** water*

The total phenolic content in the observed extracts (Fig 4.2) was assessed using the standard curve equation $y = mx + b$, which can be interpreted as "absorbance equals the slope times concentration plus the y-intercept absorbance value." This equation allowed for the quantification of phenolic compounds present in the extracts based on their absorbance values and concentration levels. The maximum emodin content of 0.227 mg/ml was observed in the Chloroform extract of Tungnath accession. The concentrations ranged from 0.178 - 0.227 mg/ml in the samples of different populations obtained via different series (Table 4.5 and Table 4.6). Depending on the kind of extract, more

especially the polarity of the solvent employed in the extraction process, the total phenolic concentration in plant extracts varies. Phenols have high solubility in polar solvents, which results in higher concentrations of these compounds in the extracts. Phenolic compounds play a crucial role in protecting plants from oxidative damage, and they also perform similar protective functions for humans.

b) Thin layer Chromatography

For ‘Thin Layer Chromatography (TLC)’ the standard i.e. emodin was dissolved in methanol in two concentrations. 0.2 mg/mL and 0.5 mg of emodin in 1 ml of methanol. The powdered leaf samples of *Rheum emodi* of different geographical locations were extracted with different solvents with a Soxhlet apparatus. These extracts of different solvents were then further used for TLC analysis. The process and solvent system for TLC has already been discussed in Chapter 3.

As per the Retention Factor values and spots so observed after exposing TLC sheet in iodine vapors the Chloroform extract of Pothivasa, the Chloroform extract of Tungnath, the Acetone extract of Dugalbitta and Chloroform extract of Chopta showed promising results thereby confirming the presence of emodi in our samples. The Rf value of standard emodin ranged from 0.6 cm of standard 1 having 0.2 mg of emodin, and standard 2 with Rf vale of 0.5 cm (Table 4.7). The actual concentration can be determined using more précised methods like HPLC.

Table 4.7: TLC RF value of different samples

Std 1 (0.2mg/ml emodin) Rf	Std 2 (0.5mg/ml emodin) Rf	Sample	Rf Value
0.6 cm	0.5 cm	**Garwali**	
		Chloroform	0.3 cm
		Acetone	--
		Methanol	0.4 cm
		Water	0.1 cm
0.8 cm	0.6 cm	**Shyalmi**	
		Chloroform	--
		Acetone	0.4 cm
		Methanol	--
		Water	--
0.6 cm	0.8 cm	**Pothivasa**	
		Chloroform	0.80 cm
		Acetone	0.50 cm
		Methanol	0.10 cm
		Water	0.67 cm
0.6 cm	0.5 cm	**Tungnath**	
		Chloroform	0.6 cm
		Acetone	0.3 cm
		Methanol	0.5 cm
		Water	--
0.8 cm	0.73 cm	**Baniyakund**	
		Chloroform	--
		Acetone	0.58 cm
		Methanol	0.35 cm
		Water	--
0.6 cm	0.33 cm	**Dogalbitta**	
		Chloroform	0.56 cm
		Acetone	0.37 cm
		Methanol	--
		Water	--
0.4 cm	0.32 cm	**Chopta**	
		Chloroform	0.32 cm
		Acetone	0.1 cm
		Methanol	--
		Water	--

4.1.4 Antimicrobial Activity Test

For antimicrobial test the crude extract was then re-dissolved in 'DMSO to get 10 mg/mL' for each extract. This solution was then used for the estimation of antimicrobial activity. The mean of zone of inhibition was calculated and verification was done by using one-way ANOVA. Antimicrobial activity was evaluated against Gram +ve and Gram –ve bacteria by agar well diffusion method. All the extracts showed significant antibacterial activity and zone of inhibitionwere observed against bacteria (*S. aureus, Bacillus, Pseudomonas, Salmonella* and *E.coli.*Whereas no zone of inhibtion was observed against the fungal strains *Aspergillus niger and Aspergillus flavus*. The zone of inhbition against various bacterial strains are illustrated in Table 4.8.

The maximum antibacterial activity of ±29mm and ±25 mm was found against *S. aureus* in acetone extract of Chopta and Acetone and methanolic extract of Tungnath accessions respectively. A clear zone of inhibition was also found against *Salmonella* of ±28 mm in Chloroform extract of Tungnath . The lowest zone of inhbition (9 ± 0.5 mm) was found in the Chloroform extract of Garwali region. The plant extracts obtained from various geographical locations exhibited the highest activity against *Salmonella,* with all the plant extracts showed some measure of involvement. On the other hand, the extracts displayed the least activity against *S. aureus.* Several extracts did not show any activity against several microorganisms thereby showing their inactivity against them.

Overall all the plant extracts showed the presence of phenolic compounds. The Chloroform extract of Tungnath and Acetone extract of Chopta were found to have activity against Salmonella and had a higher concentration of emodin as compared with the other extracts. The various figures pertaining to antimicrobial activity are illustrated in Figures (4.3, 4.4, 4.5 and 4.6).

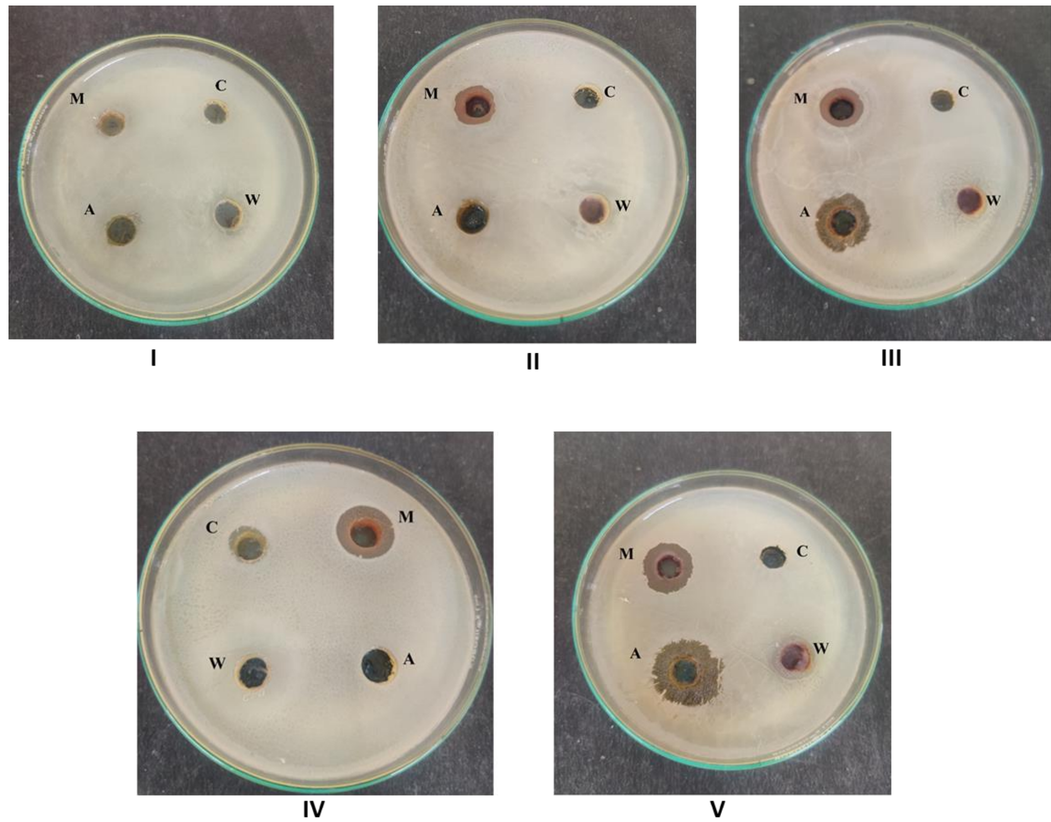


Fig 4.3: Antibacterial activity (leaf extracts) of *Rheum* against *E. coli*

(I)Control; (II) Pothivasa; (III) Baniyakund; (IV) Chopta; (V) Tungnath

Where 'A- Acetone Extract; C- Chloroform Extract; M- Methanolic Extract; W-Water Extract'

Table 4.8 : Zone of inhibition by different extracts of *Rheum* species

Sample		*Pseudomonas*	*Salmonella*	*Bacillus*	*S. aureus*	*E.coli*	*A.niger*	*A.flavus*
Garwali	C	-	9 ± 0.5	15 ± 1.1	-	16 ± 0.4	-	-
	M	-	-	-	14 ± 0.5	-	-	-
	A	-	12 ± 0.4	20 ± 0.7	-	-	-	-
	Wa	-	13 ± 0.8	-	-	-	-	-
Shyalmi	C	-	14 ± 1.7	-	-	-	-	-
	M	-	-	-	-	-	-	-
	A	10 ± 1.1	18 ± 1	-	-	-	-	-
	Wa	-	-		-	-	-	-
Pothivasa	C	12 ± 0.3	23 ± 0.6	20 ± 0.4	16 ± 0.6	-	-	-
	M	-	-	-	-	-	-	-
	A	18 ± 0.6	25 ± 1.5	22 ± 1.4	-	14 ± 0.3	-	-
	Wa	-	14 ± 0.5	-	-	-	-	-
Tungnath	C	20 ± 1.1	28 ± 0.5	15.5 ± 1	25 ± 0.3	20 ± 0.7	-	9 ± 0.5
	M	-	-	-	-	-	-	8 ± 0.5
	A	-	23 ± 1.4	16 ± 0.4	25 ± 1	18 ± 0.7	8 ± 0.5	-
	Wa	-	12 ± 1.1	-	-	13 ± 0.4	-	-
Baniyakund	C	22 ± 1.1	19 ± 1.1	-	16 ± 0.8	17 ± 0.8	-	-
	M	-	-	-	-	-	-	-
	A	12 ± 0.6	25 ± 1	17 ± 1	23 ± 0.6	16 ± 0.7	-	-
	Wa	-	12 ± 1	-	-	-	-	-
Dogalbitta	C	18 ± 0.3	17 ± 1	-	-	-	-	-
	M	-	-	-	-	-	-	-
	A	-	16 ± 0.4	-	-	19 ± 0.3	-	-
	Wa	-	11 ± 0.4	-	-	-	-	-
Chopta	C	14 ± 0.3	20 ± 0.4	-	-	11 ± 0.7	-	-
	M	-	-	-	-	-	-	-
	A	20 ± 0.8	28 ± 2.3	20 ± 0.5	29 ± 1	19 ± 0.8	-	8 ± 0.5
	Wa	-	-	-	-	-	-	-

(-) = no activity,C - Chloroform extract, M – Methanol Extract, A – Acetone Extract, Wa – Water ExtractAll the values in mm, well diameter – 8mm

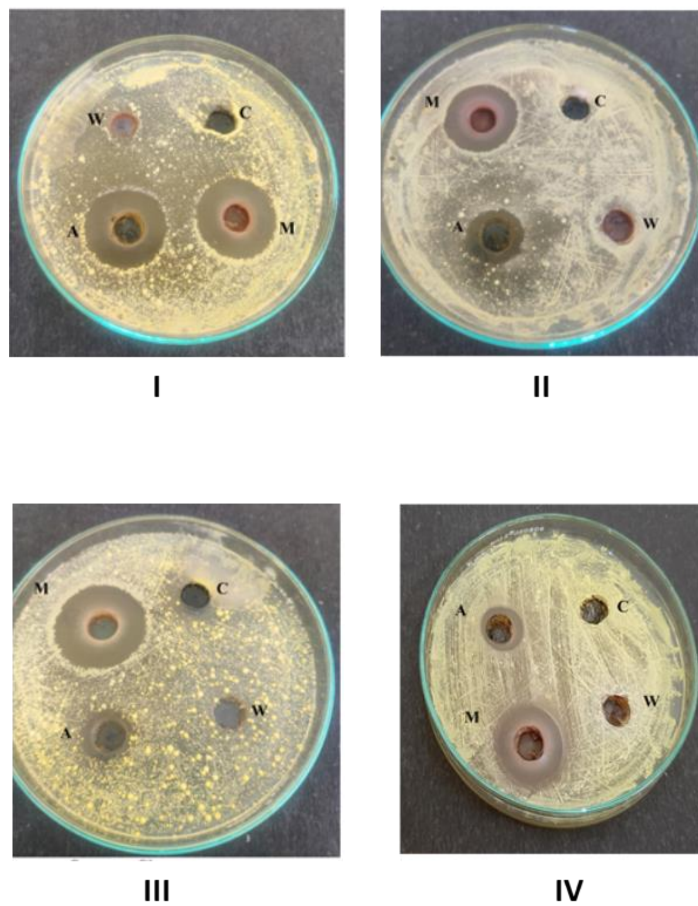


Fig 4.4: Antibacterial activity (leaf extracts) of *Rheum* against *S. aureus*

(I)Tungnath; (II) Baniyakund; (III)Chopta; (IV) Pothivasa

Where 'A- Acetone Extract; C- Chloroform Extract; M-Methanolic Extract; W-Water Extract'

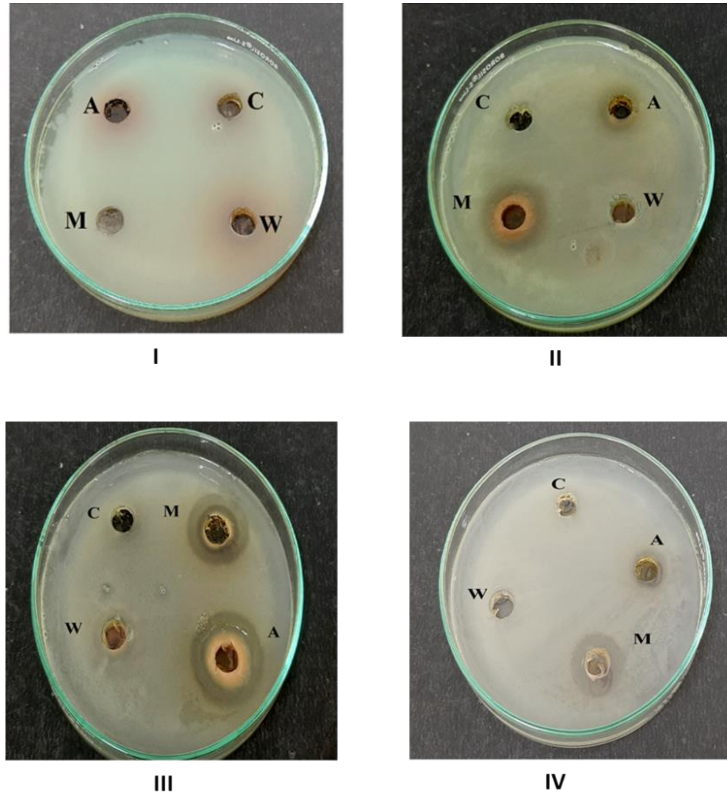
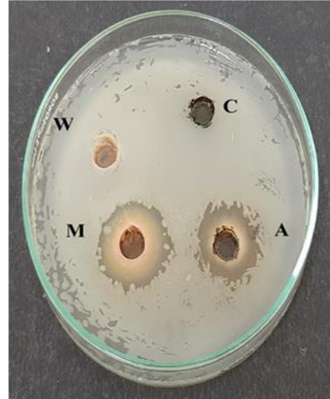


Fig 4.5: Antimicrobial activity (leaf extracts) of *Rheum* against *Pseudomonas and Salmonella*

(I)*Pseudomonas* Control; (II) Samples of Pothivasa against *Pseudomonas* (III)Samples of Pothivasa against *Salmonella*; (IV) Samples of Shyalmi against *Salmonella;* where 'A- Acetone Extract; C- Chloroform Extract; M- Methanolic Extract; W-Water Extract'



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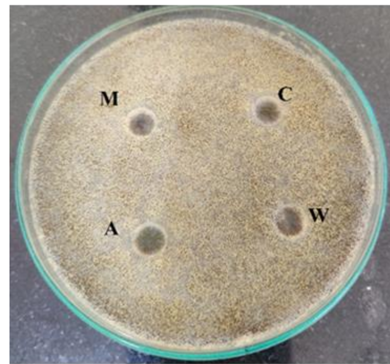

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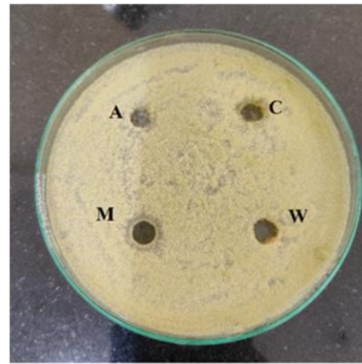

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Fig 4.6: Antimicrobial activity (leaf extracts) of *Rheum* against *Bacillus and Aspergillus niger and Aspergillus flavus*

(I)Plant extracts of Pothivasa against *Bacillus;* (II) and (III) are plant extracts samples of Tungnath against *Aspergillus niger and Aspergillus flavus*; where A- Acetone Extract; C- Chloroform Extract; M- Methanolic Extract; W-Water Extract

4.2 Standardization of protocol for Isolation of Genomic DNA

4.2.1 DNA Extraction

The Genomic DNA was isolated using a modified CTAB method with few modifications in modified protocol of Doyle and Doyle, (1990),[150] DNA was visualized in 0.8% Agarose gel. The isolated DNA was found suitable for the extraction of desired quantity and quality of genomic DNA. The bands showed clear and sharp visible DNA bands (Fig 4.7).

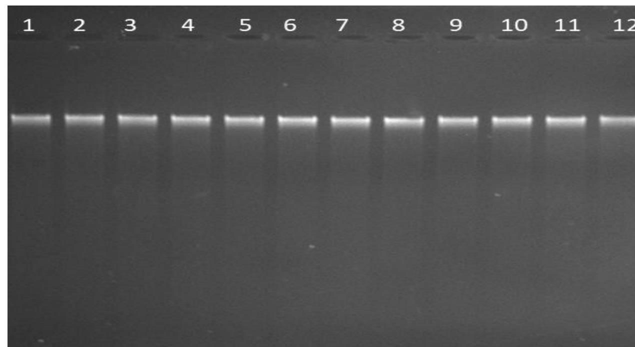

Fig 4.7: Gel image of isolated genomic DNA from leaf tissues of *Rheum* species

4.2.2 Quantification of Isolated DNA

The DNA concentration is determined by measuring the absorbance of the sample at 260nm and then multiplying it by the dilution factor.

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

The ratio of A_{260} and A_{280} for most of the samples were evaluated in the range of 1.6 – 1.9, thereby confirming the purity of DNA (Table 4.9 (i - ix)). The concentration of DNA was found to be in the range of 655 – 1445 ng/µl. The purity of DNA refers to the degree to which the DNA sample is free from impurities, such as 'proteins, RNA, and other contaminants'. For the determination of purity of DNA, the ratio of absorbance at 260 nm

and 280 nm (A260/A280 ratio) is calculated. It is an indicator of the presence of contaminants that may absorb light at these wavelengths. A high A260/A280 ratio (usually around 1.8) specifies that the DNA sample is pure, while a low ratio suggests contamination with other molecules. It is important to ensure that DNA samples are pure before using them for downstream applications, such as PCR, sequencing, and cloning, as contaminants can interfere with these processes and lead to inaccurate results.

Table 4.9: Concentration and Absorbance ratio of genomic DNA extracted from 9 different landraces of *Rheum* species

i. Population 1 – Pothivasa

Sample No.	Absorbance 260 (nm)	Absorbance 280 (nm)	Absorbance Ratio 260/280	Concentration (ng/µl)
1.	0.288	0.165	1.74	1440
2.	0.273	0.142	1.92	1365
3.	0.243	0.151	1.60	1215
4.	0.250	0.144	1.73	1250
5.	0.178	0.102	1.70	890
6.	0.219	0.120	1.80	1095
7.	0.158	0.099	1.60	790
8.	0.223	0.132	1.68	1115
9.	0.182	0.101	1.80	910
10.	0.215	0.124	1.73	1075
11.	0.219	0.125	1.75	1095
12.	0.265	0.135	1.96	1325

ii. Population 2– Triyuginarayan

Sample No.	Absorbance 260 (nm)	Absorbance 280 (nm)	Absorbance Ratio 260/280	Concentration (ng/µl)
1.	0.184	0.101	1.82	920
2.	0.175	0.092	1.91	875
3.	0.167	0.086	1.95	835
4.	0.289	0.152	1.90	1445
5.	0.231	0.142	1.62	1155
6.	0.243	0.148	1.64	1215
7.	0.235	0.141	1.66	1175
8.	0.247	0.135	1.82	1235
9.	0.239	0.148	1.61	1195
10.	0.211	0.125	1.68	1055
11.	0.203	0.124	1.63	1015
12.	0.185	0.098	1.88	925

iii. Population 3 – Shyalmi

Sample No.	Absorbance 260 (nm)	Absorbance 280 (nm)	Absorbance Ratio 260/280	Concentration (ng/µl)
1.	0.236	0.141	1.67	1180
2.	0.228	0.135	1.68	1140
3.	0.270	0.143	1.88	1350
4.	0.272	0.152	1.78	1360
5.	0.287	0.169	1.69	1435
6.	0.259	0.142	1.82	1295
7.	0.206	0.120	1.71	1030
8.	0.238	0.141	1.68	1190
9.	0.279	0.150	1.86	1395
10.	0.233	0.140	1.66	1165
11.	0.239	0.149	1.60	1195
12.	0.245	0.142	1.72	1225

iv. Population 4 - Tungnath

Sample No.	Absorbance 260 (nm)	Absorbance 280 (nm)	Absorbance Ratio 260/280	Concentration (ng/µl)
1.	0.252	0.134	1.88	1260
2.	0.276	0.163	1.69	1380
3.	0.276	0.146	1.89	1380
4.	0.263	0.152	1.73	1315
5.	0.281	0.172	1.63	1405
6.	0.256	0.135	1.89	1280
7.	0.237	0.138	1.71	1185
8.	0.274	0.165	1.66	1370
9.	0.240	0.142	1.69	1200
10.	0.246	0.127	1.93	1230
11.	0.207	0.128	1.61	1035
12.	0.252	0.134	1.88	1260

v. Population 5 – Ravanshila

Sample No.	Absorbance 260 (nm)	Absorbance 280 (nm)	Absorbance Ratio 260/280	Concentration (ng/µl)
1.	0.278	0.152	1.82	1390
2.	0.167	0.098	1.70	835
3.	0.281	0.152	1.84	1405
4.	0.197	0.107	1.84	985
5.	0.234	0.123	1.90	1170
6.	0.213	0.129	1.65	1065
7.	0.136	0.073	1.86	680
8.	0.190	0.106	1.79	950
9.	0.283	0.160	1.76	1415
10.	0.207	0.121	1.71	1035
11.	0.230	0.141	1.63	1150
12.	0.254	0.145	1.75	1270

vi. Population 6 - Chopta

Sample No.	Absorbance 260 (nm)	Absorbance 280 (nm)	Absorbance Ratio 260/280	Concentration (ng/µl)
1.	0.231	0.133	1.73	1155
2.	0.225	0.132	1.70	1125
3.	0.213	0.125	1.70	1065
4.	0.245	0.142	1.72	1225
5.	0.165	0.091	1.81	825
6.	0.287	0.176	1.63	1435
7.	0.140	0.081	1.72	700
8.	0.161	0.092	1.75	805
9.	0.139	0.078	1.78	695
10.	0.248	0.149	1.66	1240
11.	0.276	0.162	1.70	1380
12.	0.265	0.150	1.77	1325

vii. Population 7 - Dugalbitta

Sample No.	Absorbance 260 (nm)	Absorbance 280 (nm)	Absorbance Ratio 260/280	Concentration (ng/µl)
1.	0.210	0.125	1.68	1050
2.	0.234	0.123	1.90	1170
3.	0.253	0.152	1.66	1265
4.	0.141	0.085	1.65	705
5.	0.281	0.163	1.72	1405
6.	0.236	0.131	1.80	1180
7.	0.247	0.138	1.78	1235
8.	0.201	0.118	1.70	1005
9.	0.265	0.141	1.87	1325
10.	0.230	0.136	1.69	1150
11.	0.178	0.102	1.70	890
12.	0.131	0.073	1.79	655

viii. **Population 8 - Baniyakund**

Sample No.	Absorbance 260 (nm)	Absorbance 280 (nm)	Absorbance Ratio 260/280	Concentration (ng/µl)
1.	0.281	0.172	1.63	1405
2.	0.213	0.121	1.76	1065
3.	0.273	0.141	1.93	1365
4.	0.193	0.098	1.96	965
5.	0.263	0.132	1.99	1315
6.	0.232	0.141	1.64	1160
7.	0.181	0.106	1.70	905
8.	0.255	0.152	1.67	1275
9.	0.163	0.095	1.71	815
10.	0.223	0.136	1.63	1115
11.	0.263	0.142	1.50	1315
12.	0.278	0.152	1.82	1390

ix. **Population 9 – Garwali**

Sample No.	Absorbance 260 (nm)	Absorbance 280 (nm)	Absorbance Ratio 260/280	Concentration (ng/µl)
1.	0.224	0.115	1.94	1120
2.	0.287	0.162	1.77	1435
3.	0.135	0.076	1.77	675
4.	0.216	0.128	1.68	1080
5.	0.234	0.140	1.67	1170
6.	0.186	0.103	1.80	930
7.	0.229	0.138	1.65	1145
8.	0.274	0.152	1.80	1370
9.	0.255	0.142	1.79	1275
10.	0.241	0.135	1.78	1205
11.	0.271	0.141	1.92	1355
12.	0.246	0.152	1.61	1230

4.2.3 Dilution Preparation

The concentration of DNA used in the present study is 20 ng/µl. The dilutions were prepared using the formula $C_1V_1 = C_2V_2$. It is important to dilute the samples to bring them to a common concentration range before proceeding to PCR analysis (Table 4.10).

Table 4.10: Dilutions of Genomic DNA samples

Sr No	Conc. of Genomic DNA(ng/µL)	DNA (µL)	De-ionized Water (µL)
1.	1440	13.8	986
2.	1365	14.6	985
3.	1215	16.4	984
4.	1250	16	984
5.	890	22.4	977
6.	1095	18.2	981
7.	790	25.3	974
8.	1115	17.9	982
9.	910	21.9	978
10.	1075	18.6	981
11.	1095	18.2	982
12.	1325	15.0	985

4.3 Molecular Marker Analysis

4.3.1 Random Amplified Polymorphic DNA (RAPD) Analysis

PCR was performed with RAPD primers from Sigma Aldrich. 15 primers were screened initially for obtaining reproducible and distinct bands. Out of them only twelve primers produced acceptable results and reproducibility and these were used to amplify 108 individuals of 9 landraces of *Rheum emodi* species. The PCR reactions were performed with greatest precision to evaluate diversity among the populations.

4.3.1.1 Assessment of Genetic Diversity among population

The current study is focused on the assessment of genetic diversity and population structure among the population. The genetic data analyze for 9 different landraces of *Rheum emodi* is given in Table 4.11. The highest value of haplotypic diversity was observed in TG i.e. Tungnath sample (h = 0.31) and the lowest in SM (Shyalmi) h = 0.21. The estimates of the effective number of haplotypes or alleles (ne) and haplotypic diversity or Nei genetic diversity (h) averaged across all the populations were 1.45 and 0.25 respectively whereas the observed no. of alleles (na) averaged to 1.65. The 'average effective no. of alleles' (ne) per locus was 1.45.

Table 4.11: Genetic Variability within the populations of *Rheum emodi* obtained from RAPD analysis

Population Code	Ss	na*	ne*	h*
PV	12	1.63	1.39	0.23
TN	12	1.67	1.45	0.25
SM	12	1.57	1.37	0.21
TG	12	1.78	1.55	0.31
RS	12	1.73	1.5	0.28
CH	12	1.59	1.44	0.24
DG	12	1.59	1.45	0.25
BK	12	1.67	1.51	0.28
GW	12	1.64	1.46	0.26
Average	12	1.65	1.45	0.25

*'Ss – Sample size, * ne = Effective number of alleles [Kimura and Crow (1964)],[159] * na = Observed number of alleles, * h = Nei's (1973) gene diversity'[160]*

Table 4.12: Genetic Diversity analysis indicators for nine populations of *Rheum emodi*

Population Code	Ss	Np	PPB (%)	h*	I*	Average G_{ST} among Population	Average N_m among Population
PV	12	90	63.38	0.23	0.34	0.3213	1.05
TN	12	96	67.61	0.25	0.37		
SM	12	81	57.04	0.21	0.31		
TG	12	112	78.87	0.31	0.45		
RS	12	104	73.24	0.28	0.41		
CH	12	84	59.15	0.24	0.35		
DG	12	84	59.15	0.25	0.36		
BK	12	96	67.61	0.28	0.41		
GW	12	92	64.79	0.26	0.37		
Average	12	93.2	65.64	0.25	0.37		

*Ss = sample size, Np = The number of polymorphic loci, * h = Nei's (1973) gene diversity,[160] * I = Shannon's Information index [Lewontin (1972)][161], PPB = The percentage of polymorphic loci, G_{ST} = Genetic differentiation among populations, N_m = Estimated gene flow*

The percentage of polymorphic loci or bands ranged from 59.15 – 78.87 % with an average of 65.64 % at the population level in *Rheum* species. Assuming Hardy-Weinberg equilibrium, Nei's gene diversity (h) varied between 0.21 – 0.31 with an average of 0.25. The "Shannon's Information index (I)" ranged from 0.31 – 0.45 with an average of 0.37. In *R. emodi*, a total of 65.64% polymorphic fragments were observed (as shown in Table 4.12). At the population level, samples from population TG exhibited the highest number of polymorphic bands, accounting for 78.87% of the total. The populations of this landrace, when considered collectively, displayed a "Nei's genetic diversity" value of 0.31 and a Shannon's Diversity index of 0.45. In contrast the population SM showed the least "percentage of polymorphic bands" (57.04 %) with "Nei's genetic diversity" of 0.21 and "Shannon's Diversity index" of 0.31.

4.3.1.2 RAPD Polymorphism

A total of 12 primers were used in the amplification process, resulting in the amplification of 142 fragments. On average, each primer amplified approximately 11.8 fragments. The sequences of primer are given in Table 4.13. All the primers generated a total of 142 primers with an average of 65.64 % polymorphic loci. Maximum fragments (14) were amplified by OPA 09 and OPB 04 while minimum fragments (9) were amplified by 2 primers OPL 10 and OPP 11 whereas the mean per fragment was 11.8.

Table 4.13: Characteristics of 12 RAPD primers for *Rheum* species

Sr no	Oligo Name	Tm (⁰C)	Sequence (5`- 3`)	No. of Bands	Range of Amplification (bp)	PIC
1	OPA 09	37	GGGTAACGCC	14	200 - 3000	0.38
2	OPB 09	37	TGGGGGACTC	12	200 - 2000	0.39
3	OPE 06	37	AAGACCCCTC	12	400 - 2000	0.38
4	OPL 10	31	TGGGAGATGG	09	500 - 1500	0.39
5	OPL 19	37	GAGTGGTGAC	13	300 - 1500	0.39
6	OPP 11	30	AACGCGTCGG	09	400 - 1500	0.39
7	OPP 12	37	AAGGGCGAGT	12	200 - 1500	0.41
8	OPA 17	27	GACCGCTTGT	11	200 - 2000	0.39
9	OPA 19	37	CAAACGTCGG	13	500 - 3000	0.40
10	OPB 04	37	GGACTGGAGT	14	500 - 1500	0.39
11	OPE 08	31	TCACACGGT	10	500 - 2000	0.40
12	OPD 04	35	TCTGGTGAGG	13	300 - 1500	0.43
Total				142	100 - 3000	
Mean				11.8		0.39

Tm = Annealing temperature, PIC = Polymorphism information content

Representative RAPD profile generated by OPA 09, OPB 04, OPE 08, OPD 04, OPA 19 are shown by Figure 4.8 (a), (b), (c), (d), (e) respectively. The polymorphic information content (PIC) varied from 0.38 (OPA 09, OPE 06) to 0.43 (OPD 04) with an average of 0.39. However most of the primers showed a PIC value of 0.39 (Table 4.13).

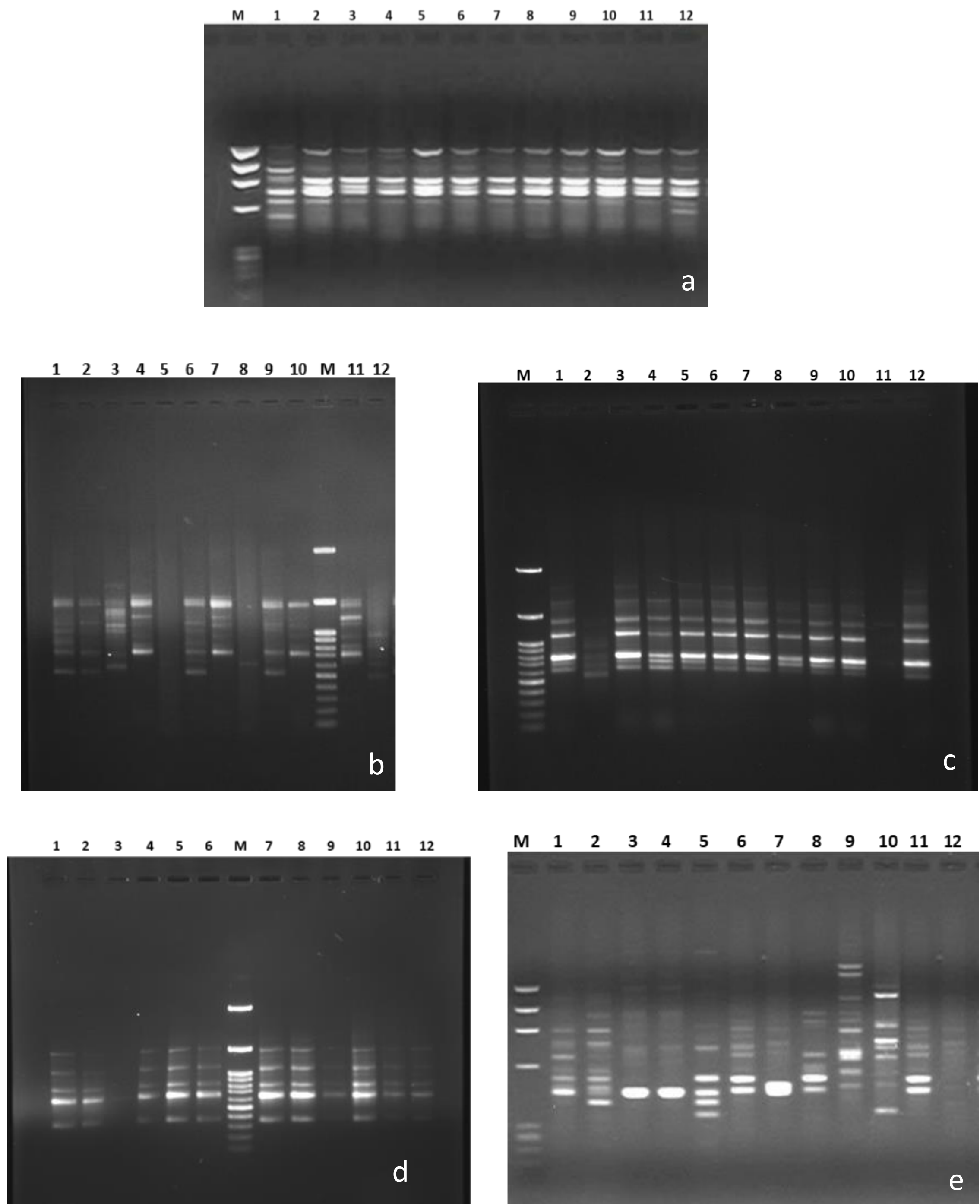

Fig 4.8: Polymorphic RAPD profile of different landraces amplified by (a) OPA 09, (b) OPB 04, (c)OPE 08, (d) OPD 04, (e) OPA 19

4.3.1.3 Genetic Variability and Analysis of Molecular Variance (AMOVA)

Significant genetic differentiation was observed among the populations of *Rheum* species. The coefficient of 'Genetic Differentiation (Gst)' was calculated to be 0.3213, indicating that only 32.13% of the genetic variability was distributed among populations, which is relatively low (as shown in Table 4.12). The level of gene flow (Nm) was found to be 1.05 individuals per population.

According to the AMOVA analysis, there were significant ($P < 0.05$) genetic differences among the populations. Among the total genetic diversity, only 5.04% was attributed to differences among the populations, while the majority (94.96%) was due to differences within the populations (as shown in Table 4.14). The AMOVA results suggest that there is considerable variation among individuals of the same species. This could be one of the reasons for declining diversity of *Rheum* species. The AMOVA (F_{ST}) was found to be 0.05 indicating low levels of genetic differentiation among populations.

Table 4.14: AMOVA of RAPD data of different populations of *Rheum* species

Source of Variation	Degree of freedom	Sum of squares	Variance Components	Percentage of variation	P value *	Fixation Index (Fst)
Among Population	8	74.14	0.3	5.04	<0.05	0.05
Within Population	99	560.5	5.66	94.96	<0.05	
Total	107	634.64	5.96			

**Significance tests after 1023 permutations*

4.3.1.4 Genetic Distance

The genetic distance was calculated using Nei's (1972) unbiased measures of genetic distance among populations using Popgene software. Table 4.15 shows the genetic distance calculated between populations for different population of *Rheum* species. Based on the table, the lowest genetic distance observed was 0.1093, which was between the Dugalbitta and Baniyakund populations. On the other hand, the highest genetic distance recorded was 0.3353, which was between the Pothivasa and Tungnath populations. The distances calculated in the study correspond to the actual geographic allocations and reflect the genetic distances observed in the table. However, Pothivasa and Tungnath are not in vicinity with each other this can be due to the less number of sampled populations.

4.3.1.5 Cluster Analysis

RAPD based dendrogram of 9 populations formed two main cluster named Cluster 1 and Cluster 2 (Fig 4.9). Cluster 1 consisted of one population PV belonging to low range of altitude. Cluster 2 is again subdivided into 10 sub – clusters, 'Cluster 3, Cluster 4, Cluster 5, Cluster 6, Cluster 7, Cluster 8, Cluster 9, Cluster 10, Cluster 11 and Cluster 12'. Cluster 4 comprise of single population SM collected from low altitude range. Cluster 3 is again subdivided into sub-clusters Cluster 5 and Cluster 6 where the latter one is represented by the populations TN collected from high altitude range. The genetic distances are given in table 4.15. The cluster is again separated into cluster 7 and Cluster 8. Cluster 7 is represented by the populations TG and RS belonging to the samples collected from medium and high altitude range respectively. Cluster 8 is again subdivided into Cluster 9 and Cluster 10 represented by CH, DG, BK and GW belonging to medium altitude range populations. The analysis of grouping pattern of this cluster for the genotypes varies considerably with their geographical regions.

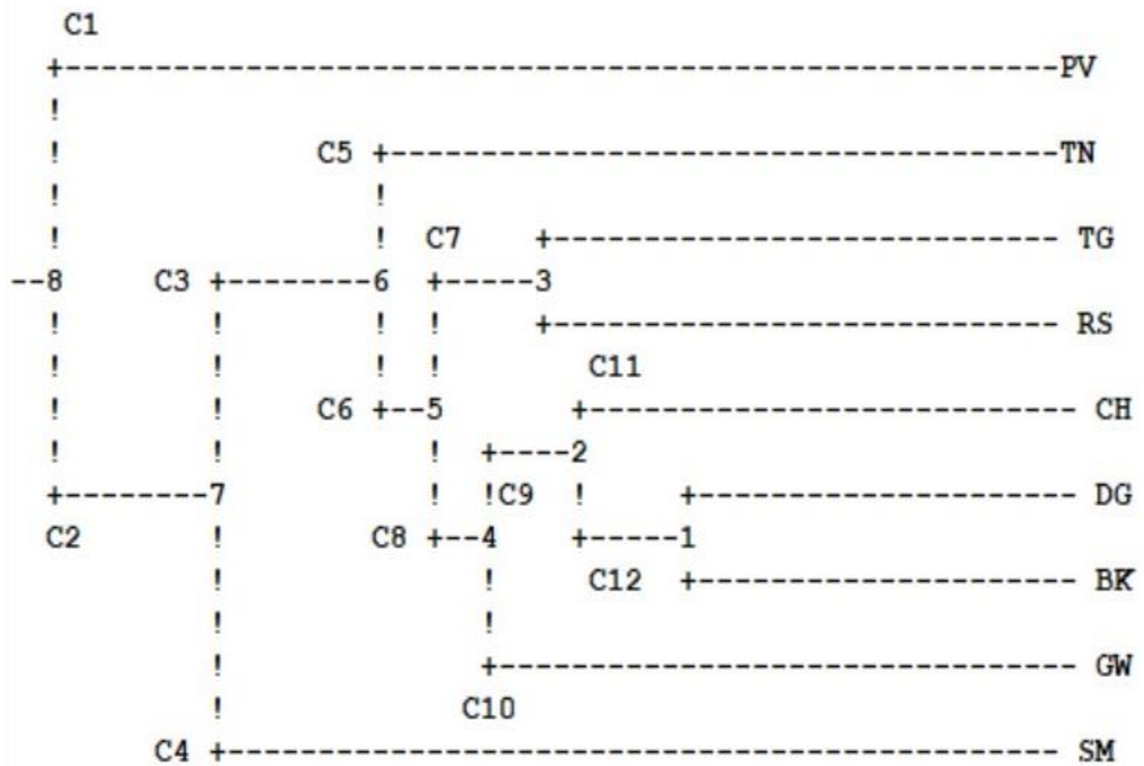

Fig 4.9: UPGMA Dendrogram for *Rheum* Species Landraces Using RAPD Marker Analysis and 'Nei's Genetic Diversity Coefficient'.

'Bootstrap Analysis with 100 Samples'

Table 4.15: 'Nei's (1972)[162] unbiased measures of genetic distance (below diagonal) among populations'

Populations	PV	TN	SM	TG	RS	CH	DG	BK	GW
PV	****								
TN	0.3353	****							
SM	0.2780	0.2136	****						
TG	0.3233	0.2063	0.2782	****					
RS	0.2878	0.1921	0.2488	0.146	****				
CH	0.2699	0.1800	0.2212	0.2237	0.1567	****			
DG	0.2280	0.1739	0.1875	0.2076	0.1373	0.1311	****		
BK	0.2503	0.1847	0.2583	0.1901	0.1603	0.1473	0.1093	****	
GW	0.2629	0.2107	0.2413	0.1731	0.1711	0.1738	0.1774	0.142	****

4.3.2 Inter Simple Sequence Repeats (ISSR) Analysis

20 primers were initially screened for PCR amplification out of which thirteen primers were selected for additional analysis. The 13 primers that could amplify visible bands and generated strong and reproducible banding patterns, were used in all samples of different populations of *Rheum* species.

4.3.2.1 Genetic Diversity among population

Out of the 108 individuals belonging to nine populations of *Rheum emodi*, a total of 13 primers resulted in 189 clearly identifiable bands ranging in size from 300 – 3000 bp, corresponding to 14.5 bands per primer. Among 9 populations from different altitudes, the average heterozygosity (h) was found to be 0.25. Assuming "Hardy- Weinberg equilibrium", the "effective number of alleles (ne)" within the population was estimated to be 1.45. Within the population level, the "PPB i.e. the percentage of polymorphic alleles" ranged from 44.97 % - 79.37 % with an average of 62.84%. The "Nei's genetic diversity" (h) shown by the population of *Rheum* species showed an aggregate range of 0.17 – 0.30 with a combined average of 0.25. The "Shannon's index (I)" comes out to be 0.25 - 0.44 within the population with an average of 0.36. Among the studied populations, population TG displayed the highest GD with a value of h = 0.30 and I = 0.44. Conversely, population GW exhibited the lowest GD, with a value of h = 0.17 and I = 0.25. Among the population, average gene diversity and the Shannon's index was estimated to be 0.25 and 0.36 demonstrating a relatively high level of genetic diversity (Table 4.16).

4.3.2.2 Genetic Variability

Based on the results obtained from "Nei's genetic diversity and AMOVA analysis", significant genetic differentiation was observed among the populations of *R. emodi*. The "genetic differentiation among populations (GST)" was estimated to be 0.29, indicating that only 29.96% (approximately 30%) of the genetic variability was distributed among the populations, which is relatively low (Table 4.16). These results are in accordance with

the results obtained from RAPD analysis. The value of gene flow (N_m) is quite high 1.16 thereby confirming low genetic differentiation.

Table 4.16: Genetic Variability within the populations of *Rheum emodi* obtained from ISSR analysis

Population Code	Ss	na*	ne*	h*	I*	Np	PPB (%)	Average G_{ST} among Population	Average N_m among Population
PV	12	1.51	1.39	0.21	0.31	98	51.85	0.2996	1.169
TN	12	1.6	1.43	0.24	0.35	115	60.85		
SM	12	1.56	1.42	0.23	0.34	107	56.61		
TG	12	1.79	1.53	0.30	0.44	150	79.37		
RS	12	1.73	1.52	0.29	0.43	139	73.54		
CH	12	1.69	1.5	0.28	0.41	132	69.84		
DG	12	1.67	1.49	0.27	0.39	127	67.20		
BK	12	1.61	1.49	0.26	0.38	116	61.38		
GW	12	1.44	1.3	0.17	0.25	85	44.97		
Average	12	1.62	1.45	0.25	0.36	118.7	62.84		

** h = Nei's (1973) gene diversity, * I = Shannon's Information index [Lewontin (1972)][161], Ss = sample size, *na = Observed number of alleles, * ne = Effective number of alleles [Kimura and Crow (1964)][159], Np = The number of polymorphic loci, PPB = The percentage of polymorphic loci, G_{ST} = Genetic differentiation among populations, N_m = Estimated gene flow*

4.3.2.3 ISSR Polymorphism

Thirteen primers were carefully selected for the study of GD in *R. emodi* landraces based on various criteria, including: "the number of amplification products, the quality of profiles, the level of polymorphism, and the reproducibility of the bands". In total, the 13 primers amplified 189 fragments, resulting in an average of 14.5 fragments per primer. Among the primers used, UBC 808 generated the maximum number of fragments (20), while two primers, UBC 842 and UBC 811, amplified the minimum number of fragments (11 each).

Further analysis of the primers revealed a range of "polymorphic information content (PIC) values", with diversity indices ranging from 0.36 to 0.42 (as shown in Table 4.17) with maximum number of primers with a value of 0.42 as PIC. The representatives ISSR profiles are illustrated in Fig 4.10.

Table 4.17: Characteristics of 13 ISSR primers utilized to assess the genetic diversity in *Rheum* species

Sr no	Primer Name	Tm (^{0}C)	Primer Sequence (5`-3`)	No. of Bands	Range of Amplification (bp)	PIC
1	UBC 834	52.7	AGAGAGAGAGAGAGAGCTT	14	300 - 1500	0.42
2	UBC 835	52.7	AGAGAGAGAGAGAGAGCTC	13	200 - 2000	0.42
3	UBC 842	52.7	GAGAGAGAGAGAGAGACTG	11	500 - 1500	0.41
4	UBC 811	43.3	GAGAGAGAGAGAGAGAC	11	400 - 1500	0.43
5	UBC 808	47.1	AGAGAGAGAGAGAGAGC	20	200 - 1500	0.36
6	UBC 809	48.7	AGAGAGAGAGAGAGAGG	15	200 - 2000	0.42
7	UBC 868	48.7	GAAGAAGAAGAAGAAGAA	16	100 - 3000	0.40
8	UBC 840	47.1	GAGAGAGAGAGAGAGACTT	15	200 - 2000	0.42
9	UBC 841	60.0	GAGAGAGAGAGAGAGACGACTC	15	200 - 2000	0.40
10	UBC 889	59.2	AGTCGTAGTACACACACACACAC	18	300 - 1200	0.38
11	UBC 891	64.0	AGTACGAGTTGTGTGTGTGTG	12	200 - 1500	0.42
12	UBC 890	67.0	ACGAGTAGGGTGTGTGTGTGTGT	13	200 - 3000	0.39
13	UBC 888	67.0	CTGAGTCGTCACACACACACACA	16	200 - 2000	0.41
Total				189	100 - 3000	
Mean				14.5		0.40

(Tm = Annealing temperature, PIC = Polymorphism information content)

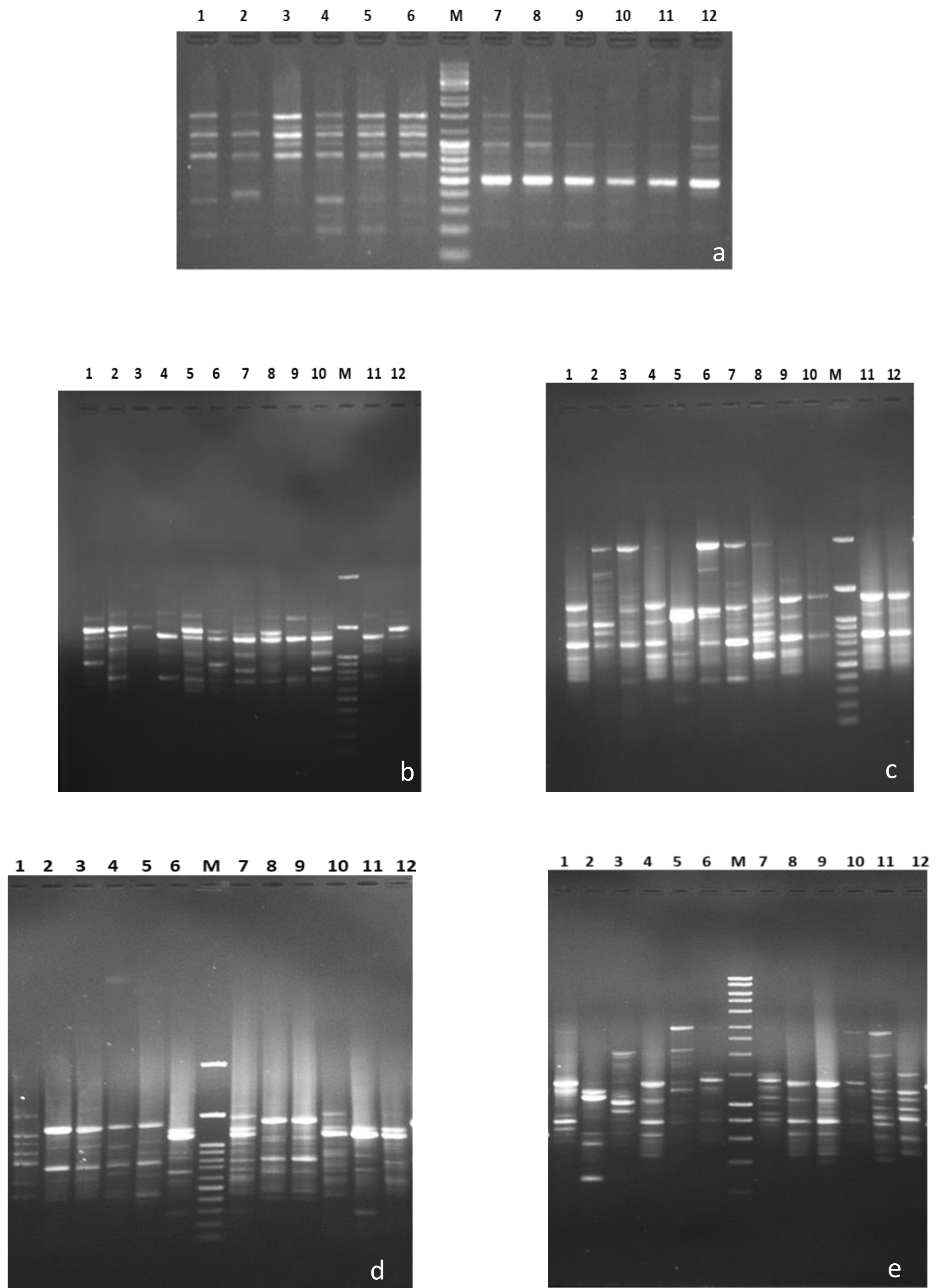

Fig 4.10: Polymorphic ISSR profile of different landraces amplified by (a) UBC 808, (b) UBC 842, (c) UBC 868, (d) UBC 834, (e) UBC 841

4.3.2.4 Genetic Relationship

'The UPGMA dendrogram based on Nei's (1972) unbiased genetic distance matrix'[162] (Table 4.19) by using 13 ISSR markers were constructed. The dendrogram segregated the nine populations into two main clusters (Cluster 1 and Cluster 2), the genetic distance coefficients ranges between 0.2629 (TN and CH) to 0.1314 (PV and GW). Cluster 1 comprises of eight populations (Fig 4.11) PV, TG, GW, TN, SM, CH, DG and BK) while cluster 2 consist of single population of RS obtained from high altitudinal range. Cluster 1 is again sub-divided into two clusters, Cluster 3 and Cluster 4. Cluster 4 is divided into two sub clusters (Cluster 5 and 6). Cluster 6 comprised of only one population BK, while Cluster 5 comprised of two populations CH and DG of medium to low altitudinal range.

Cluster 3 comprises of five populations which are again subdivided into two clusters (Cluster 7 and cluster 8). Cluster 7 comprise of populations TN and SM whereas Cluster 8 is divided into two sub- clusters C9 (PV) and C10. TG and GW were residing in Cluster 10. RS in Cluster 2 was placed specially from the other ones indicating that the population has different genetic makeup from others.

4.3.2.5 Analysis of Molecular Variance (AMOVA)

The AMOVA test confirmed that differentiation among populations was statistically significant ($p < 0.05$, as shown in Table 4.18). Out of the total molecular variance, 2.7% was attributed to diversity among populations, while the majority (97.30%) was observed within the populations. This indicates that most of the genetic variation is contained within each population, with only a small portion of the variance being attributed to differences among populations. The AMOVA (F_{ST}) was found to be 0.27 indicating low levels of genetic differentiation among populations.

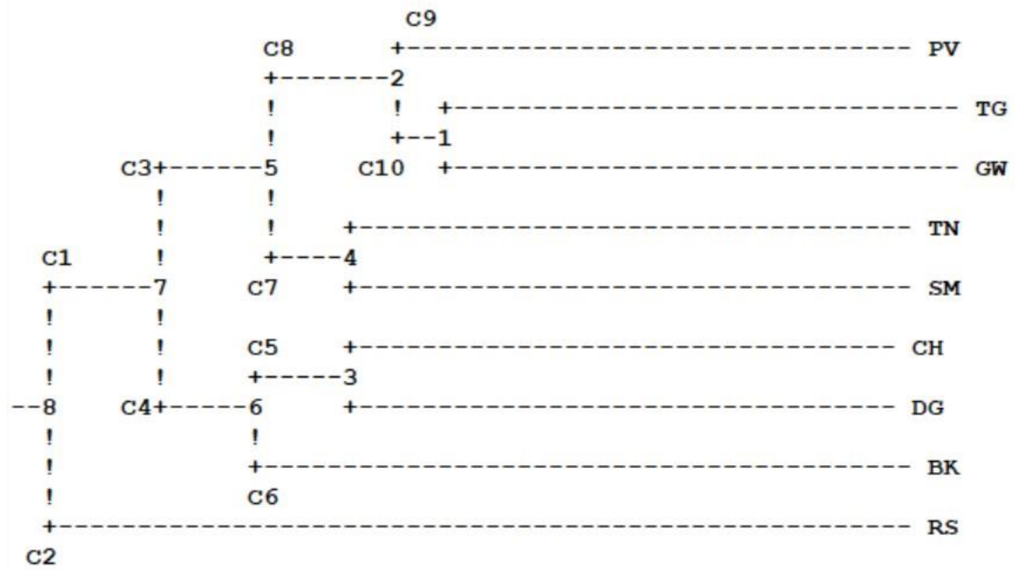

Fig 4.11: UPGMA Dendrogram for *Rheum* Species Landraces Using ISSR Marker Analysis and 'Nei's Genetic Diversity Coefficient. Bootstrap Analysis with 100 Samples'

Table 4.18: Results of analysis of molecular variance (AMOVA) of ISSR data from 9 landraces of *Rheum species*

Source of Variation	**Degree of freedom**	**Sum of squares**	**Variance Components**	**Percentage of variation**	**P value ***	**Fixation Index (Fst)**
Among Population	8	67.20	0.17	2.7	<0.05	0.2701
Within Population	99	623.83	6.30	97.30	<0.05	
Total	107	691.03	6.47			

Table 4.19: 'Nei's (1972) unbiased measures of genetic distance (below diagonal) among populations'

Populations	PV	TN	SM	TG	RS	CH	DG	BK	GW
PV	****								
TN	0.1427	****							
SM	0.1803	0.1431	****						
TG	0.1481	0.1811	0.1810	****					
RS	0.1881	0.2347	0.2583	0.1538	****				
CH	0.2107	0.2629	0.2350	0.1496	0.1764	****			
DG	0.2137	0.2184	0.1973	0.1800	0.1984	0.1414	****		
BK	0.1953	0.1694	0.1822	0.1769	0.1554	0.1917	0.1417	****	
GW	0.1314	0.1545	0.1512	0.1494	0.2045	0.1889	0.1573	0.1451	****