Chapter 5

DISCUSSION

5.1 Phytochemical Screening for active components

Plants are rich sources of diverse beneficial compounds, including glycosides, alkaloids, flavonoids, tannins, and phenolic compounds, making them a valuable source of safe and effective medicines. *Rheum,* a medicinal plant, is known for its anti-cancer and anti-oxidant properties. Its pharmacological activities are attributed to anthraquinone, a major class of phytochemicals present in its roots and rhizomes. Aloe-emodin, emodin, chrysophanol, physcion, and rhein, which are anthraquinones, have been acknowledged for their potential anti-cancer properties.[35] The anthraquinones, namely aloe-emodin, rhein, and emodin, have been shown to have anticancer activity on various types of cancer cells. Aloe-emodin has demonstrated efficacy in combating neuroectodermal tumors, lung squamous cell carcinoma, and hepatoma cells, as evidenced by a study conducted by Lee *et al.,* in 2001.[56] The existence of these phytochemicals, such as the anthraquinones mentioned earlier, largely accounts for the diverse activities exhibited by *Rheum* species.

As per the studies conducted by Chihara *et al.,* 2015, Emodin and aloe-emodin are two anthraquinone compounds found in various plants, including *Rheum.*[55] Wide-ranging research has been directed on these compounds to explore their pharmacological properties and determine their potential therapeutic applications. Emodin, in particular, possess various beneficial activities, including anti-inflammatory, anti-tumor, anti-viral, and anti-bacterial properties. Research and findings demonstrate the role of emodin in inhibiting the growth of cancer cells, such as breast cancer, colorectal cancer, and leukemia cells. Furthermore, emodin has shown potential neuroprotective effects, suggesting its potential utility in the cure of neurodegenerative disorders like Alzheimer's and Parkinson's diseases.

In the current study, a total of seven populations situated at varying altitudes in Uttarakhand were chosen as samples. These populations were subjected to screening in order to identify and assess the presence of various phytoconstituents. The initial step in standardizing a medicinal plant involves conducting a preliminary phytochemical screening. This screening enables the

identification and quantification of the bioactive compounds present in the plant. The samples collected for this study were found to exhibit the presence of alkaloids, proteins, flavonoids, carbohydrates, tannins and phenolic compounds, as well as fats and oils. Carbohydrates were absent in Garwali and Shyalmi, fixed oils were absent in most of the samples except for Shyalmi and Dugalbitta. Phenols were present in almost all of the samples from different locations, which are the important secondary metabolites responsible for anti-oxidant and anti-microbial activity of the plants. These findings are consistent with previous reports in the literature, such as those by Wani *et al*., in 2012 and Rehman *et al.,* in 2014.[75,10] Another study conducted by Aslam *et al*., in 2012 also evaluated the pharmacognostic and phytochemical properties of *R. emodi*, using HPLC to detect various constituents. These results indicate that there is no relation in between the altitude and presence or absence of phytochemicals in all the populations.[21]

In this study, a Thin Layer Chromatography (TLC) method was developed to enable the immediate quantification of emodin in the leaf extracts of *Rheum emodi*. The solvent system utilized in the TLC analysis consisted of Hexane, Chloroform, and Acetic acid in a ratio of (6:1:0.1 v/v/v). This particular solvent system successfully separated emodin from other compounds present in the extracts without any interference. To achieve optimal separation and resolution, the TLC chamber was saturated with the mobile phase for a duration of 30 minutes. This ensured good resolution of the compounds with reproducible retention factors (Rf values). For the standard concentrations of 0.2 mg/mL and 0.5 mg/mL, the Rf value of emodin was determined to be 0.6 cm±0.03 and 0.5 cm±0.03 cm, respectively. The Chloroform variants of Pothivasa, Tungnath, Chopta and the Acetone extract of Dugalbitta showed the same retention factor values as of standard thereby confirming the presence of emodin in the given samples. The results are in correlation with the Narayanan *et al.,* 2015 and Singh *et al.,* 2005 who quantified the amount of Emodin, Aloe- emodin in the *Rheum emodi* species through HPTLC. [57,88]

The standard curve for emodin in the plant extracts was linear in conc. range of 0.178 - 0.227 mg/ml in the samples of different populations obtained via different series. The maximum concentration of 0.227 mg/mL was found in the Chloroform variant of Tungnath whereas the lowest (0.178 mg / mL) in the Chloroform variant of Pothivasa. Similarly, Liu e al., in 2011 and Sharma *et al*., 2011 determined the amount of phenols using standard curve similar to our method. [163,164] Although the concentration of phenols varied and is very less as compared to literature as

the samples are from different plant species. Samarrai *et al.*, 2018 also showed the similar kind of results.[165]

Overall we can conclude that at high altitudes the concentration of emodin is high as compared to the samples belonging to a bit lower altitudes. The plants growing in high altitude regions have been found to contain higher levels of phytochemical compounds compared to those growing at lower altitudes. This is due to the harsh environmental conditions, such as low oxygen levels, cold temperatures, and intense ultraviolet radiation, which the plants have to adapt to survive. Under these conditions, oxidative stress occurs, triggering an upsurge in the generation of reactive oxygen species (ROS) within plant cells. Consequently, plants activate their defence mechanisms to counteract oxidative damage by synthesizing an increased amount of phytochemical compounds. These compounds include flavonoids, phenolic acids, and alkaloids, which act as protective agents against the detrimental effects of oxidative stress. These compounds act as antioxidants, scavenging the ROS and protecting the plant cells from damage.[166] Therefore, plants growing in high altitude regions have evolved to produce higher amounts of phytochemical compounds as a survival strategy, making them a valued source of natural antioxidants, exhibiting potential health benefits for humans.

To evaluate the antimicrobial properties of various extracts derived from *Rheum emodi*, the presence of inhibition zones was used as an indicator. The maximum antibacterial activity of ±29 mm 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 Chlororom extract of Garwali region. The plant extracts obtained from different geographical locations showed the highest level of activity against *Salmonella*, as all the tested plant extracts demonstrated some level of inhibitory effect. On the other hand, the extracts displayed the least activity against *S. aureus*, indicating a relatively lower inhibitory effect against this microorganism. Several extracts did not show any activity against several microorganisms thereby showing their inactivity against them. The antimicrobial activity of *Rheum emodi* has been attributed primarily to the compound Anthraquinone, which falls under the category of phenolic compounds. Several studies, including those conducted by Hussain *et al.* (2010) and Agarwal *et al.* (2000), have described on the antibacterial properties of *Rheum emodi*.[85,82] Additionally, Rehman *et al.*, (2014) have specifically mentioned the antimicrobial

activity of *Rheum* against *Bacillus subtilis* and *Pseudomonas aeruginosa* in their research findings.[10] The present study concluded that *Rheum* sp. extract have the great antimicrobial activity against *S. aureus*, *Bacillus.* The results show the presence of secondary metabolites and phytochemical compounds such as carbohydrate, protein, glycosides, phenols and tannins, flavonoids, steroid, terpenoids, alkaloids etc. in the crude extract of *Rheum* leaves samples. *Rheum sp*. is a rich source of medically important phytochemical. This is a preliminary study for qualitative analysis of phytochemical compounds present in *Rheum*. These phytochemicals can be used in the management of various chronic diseases.

5.2 DNA Extraction from Juvenile leaves of *Rheum* species

Genomic DNA extraction was performed using a modified CTAB (cetyltrimethylammonium bromide) method, as described by Doyle and Doyle in 1987, with few advancements using the young juvenile leaves collected from different geographical locations (Table 3.1). The DNA was visualized in 0.8% Agarose gel. 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. Suman *et al.,* 1999 and Paterson *et al*., 1993 have also testified high yield of genomic DNA using higher levels β Mercaptoethanol and NaCl to remove polysaccharides. [167,168] 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**.**[169] Clear Banding patterns with discreet bands were also obtained in RAPD and ISSR analysis. The DNA extraction protocol standardized in the study produced good quality DNA it was found suitable for molecular-based study.

5.3 Random Amplified Polymorphic DNA (RAPD) Analysis

The RAPD marker offers a cost-effective, simple and efficient approach to generating molecular information. In the present study, RAPD showed to be a promising analysis technique for the assessment of genetic variations among the populations of *Rheum* species. This tool has been already established in many other plant species for the determination of genetic diversity (Weir *et*

al., 1998 in tomato and potato,[170] Zheng *et al*., 2021 in Salvia,[171] Wahyudi *et al.*, 2020 in soybean[172] etc.)

5.3.1 Genetic Diversity among population

The presence of a wide range of genetic variations within a population can suggest that the population has undergone evolutionary changes that help them better adapt to their environment. These adaptations are crucial for the survival and success of the population, especially in situations where the environment is constantly changing. The results of this investigation suggest that there is a comparatively limited genetic variation observed within the different populations of *Rheum emodi.* Among the populations, PPB of Tungnath area was comparatively higher than the other populations. This result aligns well with a previous work on *Rheum* species done by Persson *et al.*, 2000 and Subositi *et al*., 2022.[133,135] One possible explanation for this observation is that plant species that are widely distributed across different regions need to acclimatize to a diverse range of ecological conditions, which can contribute to maintaining their huge geographical distribution. As a result, many widely distributed species tend to exhibit high levels of genetic diversity, as supported by previous studies. [173,174] Chopta and Dugalbitta were observed to have lowest PPB among the population. The genetic diversity and Shannon Index of Tungnath landrace showed Nei's genetic diversity of 0.31 and Shannon's Diversity index of 0.45. In contrast the population Shyalmi 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.

5.3.2 Population Genetic Differentiation

The population genetics study revealed noticeable genetic differentiation among the *Rheum* species populations. However, the level of genetic differentiation observed (GST = 0.32) was found to be quite low and not statistically significant. However, the gene flow value was notably higher compared to that of a single successful migrant. This considerable gene flow is a result of the interplay of several factors, including the species' evolutionary history, habitat fragmentation, mutation, gene flow, genetic drift, and selection.[175] Additionally, small populations are susceptible to genetic drift due to random sampling effects[176] and in the present study, small population of 12 sample size was employed for genetic diversity assessment. One of the reasons for extinction of *Rheum* species can be its low genetic diversity because of higher level of gene flow.

The AMOVA analysis showed the consistency with the population genetic study. The percentage variation among population and within population was 5.04 % and 94.96 %. AMOVA (F_{st}) analysis significantly revealed low levels of genetic variation occurred among populations rather than within populations. F_{ST} is directly correlated with both the degree of allele frequency variation between populations and, inversely, the degree of allele similarity within populations. The allele frequencies within each population are similar when the F_{ST} value is small, however the allele frequencies between populations are different when the F_{ST} value is large. The AMOVA analysis demonstrated that there was a significant variation attributed to differences in habitats among *Rheum emodi* populations. According to Subositi *et al.* (2022), factors such as gene flow, genetic drift, altitude, temperature, humidity, and rainfall were found to play important roles in shaping the genetic structure of these populations.[135] The limited gene flow observed may be a consequence of traditional agricultural practices, where landraces are cultivated over successive generations without the exchange of germplasms. This practice can lead to increased genetic similarity and the stabilization of specific local adaptations, as suggested by Badiane *et al*. (2004).[177]

5.3.3 RAPD Analysis

In the present study genetic diversity of *Rheum* species was assessed using RAPD markers. The RAPD technique has proven to be an efficient, straightforward, and affordable method for conducting molecular screening. It has been widely utilized in various taxonomic and phylogenetic studies, as well as in the assessment of genetic diversity (Nazarzadeh *et al.,* 2020 in *Triticum aestivum*,[178] Bi *et al.,* 2021 in *Hypericum*,[179] Yin *et al.,* 2021 in *Geranium*[180]*).*

12 primers delivered the sharp and reproducible bands. These primers differed greatly in their efficiency for revealing polymorphism. In the present study out of 15 RAPD primers, 12 primers in 108 individuals showed 65.64 % polymorphism among the populations. Primer OPA 09 showed the maximum number of bands (14) and PIC value (0.38), which is very close to PIC value of 0.4 by Ramakrishnan and his coworkers in finger millet (2016).[181] The large number of percentage polymorphism showed the high level of discriminatory power of primers. The present study and similar studies on *Rheum Officinale Baill* by Subositi *et al*., 2022,[135] Sudha *et al,* 2019 on *Allium cepa*[113] and Verma *et al*., 2017 on *Citrullus colocynthis* [125] suggested that the RAPD technique is particularly suitable for analysing genetic variability among closely related genotypes.

5.3.4 Cluster Analysis

To construct a dendrogram based on Nei's genetic distances, cluster analysis was performed using the "UPGMA (Unweighted Pair Group Method with Arithmetic Mean)" method. The most distinct genotype in the present study was Pothivasa and Shyalmi, which is isolated from rest of the populations. The lowest genetic distance calculated was 0.1093, observed between the Dugalbitta and Baniyakund populations. On the other hand, the highest genetic distance recorded was 0.3353, found between the Pothivasa and Tungnath populations. The RAPD markers used in the study grouped all the populations into 12 main clusters.

The cluster analysis showed the consistency with the AMOVA, which showed that major genetic diversity, was within the populations. The clustering of populations in the dendrogram was found to be associated with their geographic origins, although certain differences were observed. For example, populations from the same altitude were grouped into distant clusters. This finding aligns with previous studies conducted on *Oryza granulata* from China by Qian *et al*., (2001)[182] and on *Hypericum*, a medicinal plant, by Bi *et al*., (2021).[179]

Based on these observations, the genetic variations observed in this study, where populations were grouped according to both genetic distance and geographic origin, can be attributed to human activities, propagation methods, and ecological conditions.

5.4 Inter Simple Sequence Repeats (ISSR) Analysis

The 'ISSR (Inter-Simple Sequence Repeat)' technology has demonstrated high sensitivity to even minor genetic variations, making it an invaluable marker for studying population genomics in a diverse range of plant species. Additionally, it serves as a valuable tool for assessing genetic diversity among different populations. [29,136,126]

Polymorphism within a population is commonly attributed to the presence of genetic variants, which are defined by the number of alleles at a specific locus and their respective frequencies in the population. Heterozygosity, on the other hand, denotes the likelihood of randomly selecting

two alleles from the population and can be evaluated using a suitable marker. Hence, quantitative measures such as Nei's genetic diversity (h), Shannon's index (I), the coefficient of population differentiation (Gst), and estimates of gene flow provide valuable insights into the utility of a marker and the extent of polymorphism detected within a population.

5.4.1 Genetic Diversity among population

The population genetic analysis showed fairly low levels of genetic variety among the populations of *Rheum* species. The polymorphism among the nine populations of *Rheum* species was in correlation with the levels of genetic diversity as show by RAPD primers. The dominant nature of ISSR markers revealed an average percentage polymorphism of 62.84 % and percentage polymorphic band percentage ranged from 44.97 – 79.37 %. The genetic diversity among population ranged from 0.17- 0.30, which is in correspondence with similar studies done on species of *Rheum* Namely *Rheum webbianum* with a value of 0.345, followed by *R. spiciforme* with 0.241, and R. emodi with 0.237. ISSR markers were employed to evaluate the level of genetic structure and variance in five cultivated populations of *R. tanguticum.*[25,7,136] The similar pattern was shown by the Shannon Index (I), where the average Shannon index ranged from 0.25- 0.44. Our study is in resemblance with the study of Yanping Hu *et al*., (2014) on *Rheum tanguticum*, where ISSR revealed around 28.98 % intra population genetic diversity which is in fair range with our results also.[137]

The levels of genetic diversity in plant species are influenced by various factors, including breeding systems, seed dispersal methods, geographical ranges, and natural selection.[174] In the case of the studied populations, their close relationship is attributed to their collection from similar environmental conditions, characterized by comparable temperature and soil types, along with the exchange of seeds between the same regions. These geographical factors contribute to a narrow genetic base among the populations.

The landrace populations showed limited gene flow, leading to a decrease in genetic variation within the entire population. This limited genetic diversity can also contribute to the risk of extinction for plant species, especially when combined with human interventions that deteriorate the gene pool. Endangered species, in particular, tend to exhibit relatively low levels of genetic diversity, which aligns with the findings of this study.[192] In general, multiple factors play a role in

influencing genetic diversity, encompassing environmental, genetic, gene flow, genetic drift, and human factors.[183]

5.4.2 Genetic Differentiation among population

In addition to Nei's analysis of gene diversity, AMOVA analysis demonstrated that the majority of genetic variation was contained within the populations (97.30%). The genetic differentiation among the populations was relatively low, measuring at 0.2996. The genetic structure of plant populations is influenced by various processes, such as the long-term evolutionary history of the species, changes in distribution, habitat fragmentation, population isolation, mutations, gene flow, genetic drift, and selection.[29] The observed high genetic differentiation within the populations of *Rheum* may be attributed to the type of pollinators that primarily act at short distances, potentially hindering gene flow.

The low levels of genetic variations (2.7 %) among the populations can be due to geographical isolation, genetic drift, natural selection, founder effect (migration), inbreeding due to the accumulation of homozygous alleles. [184,185] The low levels of genetic variation can have negative consequences, such as reduced adaptability to changing environments or increased susceptibility to genetic disorders, high levels of genetic variation and reduced ability to fight off infections. The optimum level of genetic variability for a population can depend on numerous factors, including the environment and the genetic architecture of traits under selection.

In the present study, the effective gene flow for *Rheum species* (N_m) is quite high (1.169) thereby confirming low genetic differentiation. This interpretation is supported by the results of the cluster analysis. Populations at the same altitude tended to cluster together, aligning with their geographic distribution. These variations in the study emphasize the significance of sampling criteria in genetic diversity analysis. The samples should be chosen to represent various habitats and the species' distribution to the best possible extent. The ability of a species to adapt to its environment and ensure its long-term survival is closely tied to its genetic diversity. This diversity is an important indicator of a species' adaptive potential. In this context, the gene exchange observed in our samples, which were collected from undisturbed areas, can be seen as the outcome of early accumulation of genetic diversity same as done by Barrett *et al.*, 1991.[186]

5.4.3 ISSR marker efficiency among populations

ISSR markers are a beneficial technique for the determination of genetic variability among and within the populations of *Rheum* species. Among the populations, a polymorphism of 62.84% was observed, and a total of approximately 189 bands were amplified, with an average of 14.5 bands per primer. The detection of polymorphism can be attributed to the utilization of highly informative primers, which were identified during prior screening involving nine different populations of *Rheum* species. These markers have been successfully used to analyse the genetic diversity in *Rheum* species. [29,136,139] The effectiveness of ISSR markers relies on the presence and abundance of microsatellites, which varies across different species and the specific SSR motifs targeted. The PIC range for different primers have ben illustrated in table 4.17, where the average polymorphic content was 0.40. As per Behera and colleagues in 2008, the high level of genetic diversity observed with ISSR markers can be attributed to the considerable level of polymorphism detected.[187] ISSR markers have significant potential for unveiling both intra and inter-genomic diversity compared to other techniques based on arbitrary primers.

The present study showed that the primers with the repeated sequences AC, CA, AG and GA produced more polymorphic bands. Similar PIC values in the range of 0.5 for ISSR markers were also obtained by Ekincialp *et al.*, 2019 in *Rheum ribes* L. (wild rhubarb) in Turkey with an avg PIC value of 0.724 which is close to our study.[139]

5.4.4 Relationship among populations

A dendrogram generated from UPGMA method revealed genetic relationship among the nine populations of *Rheum* species (Fig 4.11). Genetic diversity among populations is a consequence of the interaction between genotypes and their respective environments. According to the findings of this study, it can be inferred that the distinct geographical environments play a significant role in contributing to the variations observed among the different landraces. The dendrogram segregated the nine populations into two 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.

The RAPD and ISSR derived dendrograms were not similar in comparison with each other. The dissimilar clustering of genotypes within groups observed in the dendrograms derived from RAPD and ISSR markers can be attributed to two main factors: marker sampling error and the level of polymorphism detected, as well as their coverage of the overall genome. These factors affect the accuracy of estimating the genetic relationships among cultivars. Marker sampling error refers to the inherent variability in the results obtained when randomly selecting a subset of markers from the entire pool. Due to this random selection process, different subsets of markers may yield different results, leading to variations in the clustering patterns observed within groups. [188,189]

The dendrograms generated from the data did not exhibit a distinct clustering pattern based on the geographical locations where the samples were collected. Similar results have been obtained in black gram genotype by Souframanien, J., & Gopalakrishna, T. in 2004,[189] Dwivedi *et al.*, 2001 in groundnut using RAPD marker.[190] The clustering of populations based on altitude may be influenced by various environmental factors, including temperature, rainfall, snowfall, light intensity, and UV intensity. The UPGMA cluster analysis categorized the landraces into three attitudinal ranges. This phenomenon might be related to the increase in UV intensity with altitude, leading to the generation of more genetic variation. Previous studies by Wang *et al*., (2009) supported this idea.[195] Additionally, additional plant species have also been studied for the effects of altitude and other geographic conditions on genetic diversity. [4,191] Moreover, the AMOVA analysis provides statistical support for the observed genetic structuring of the examined landraces.

This study aimed to explore population-level admixture and genetic variations among 108 individuals collected from 9 distant populations of *Rheum emodi*, an endangered plant species in the Western Himalayan region of India. The investigation utilized Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers. The study found relatively low genetic diversity at the population level (average h = 0.25) compared to the species level, as indicated by both types of markers. Analysis of molecular variance revealed minor differences among populations (5.04% and 2.7%, respectively) and a predominance of intra-population variations. Based on the genetic data obtained from this study, diverse approaches for conservation are recommended to effectively manage this endangered species. This study

represents the first report on genetic diversity and population genetic structure using RAPD and ISSR markers in *Rheum emodi* from Uttarakhand.

Therefore, the information regarding which markers is superior and the amplification reproducibility cannot be analysed. Both DNA markers (RAPD and ISSR) are suitable in the determination of genetic variability, and detection of genetically immune and strong population of *Rheum* to improve the efficacy of genotype management and conservation. Overall, the study provides valuable evidence on the genetic diversity and population genetic structure of *Rheum emodi,* which can aid in creating effective conservation strategies for this threatened species.