8. DISCUSSION

Different seed samples of *Senna obtusifolia* or the Chinese medicinal herb 'Zui Ming Zi' were collected from twenty different provenances from diverse ecogeographical regions of India as well as many districts of West Bengal and intensely analysed for different phenetic characteristics such as phenological, morphological, cytological, biochemical and genetic analyses. For its immense health benefits and awareness among consumers, marketing of the species in various forms has been greatly increased (Liu et al., 2014). Plants from all the provenances were raised in similar conditions in the experimental gardens under uniform edaphic and environmental conditions for minimizing the variations caused as a result of ecological diversity.

As the plant grows in the wild in varied conditions, variations in different phenological traits in terms of growth, flowering and fruiting patterns are quite evident of the species from different provenances. From the functional perspective of phenology, environmental variations may shed light on the response of the mechanisms of organisms (Visser et al., 2010). Phenological differences at the specific level may be a key dimension of the cause of biodiversity which also potentially promotes the coexistence of the species (Gonzalez and Loreau, 2009; Godoy and Levine, 2014; Wolkovich et al., 2014). As the species were collected from different diversified areas having changes in various factors like altitude, rainfall and temperature along with the impact of gross climate and geographical locations through years, the genetic constituents of the same species might have been affected showing different phenotypic characters.

Dinis et al., (2011) suggested that phenological and morphological diversity amongst ecotypes are due to adaptation to diverse climatic conditions and are not connected to minor genetic alterations. In the present study, *S. obtusifolia* samples belonging to diversified ecotypes report had considerable variations among the twenty-two morphological characteristics. A higher intraspecific genetic variation was observed in the populations of *S. obtusifolia* concerning the variations leading to both seed morphological traits and molecular marker polymorphism in 47 samples of diversified geographical locations of China (Mao et al., 2018).

Anthraquinone, the most important array of secondary metabolites, is quite prevalent in this species (Sob et al., 2008). The current study pioneered in isolation, identification and quantification of Rhein which is one of the simplest and immense medicinally important bioactive anthraquinones isolated from the leaves *S. obtusifolia.* The medicinal importance of Rhein is discussed in chapter 2 and chapter 6 of this thesis. HPLC methods were utilized to identify and quantify of this bioactive molecule from all the twenty accessions of the species. ESI-MS studies also confirmed the presence of Rhein from the leaf samples of *S. obtusifolia.* The higher quantity of Rhein was found in the provenances like Purulia (PRL), Tatanagar (TNG), Jabbalpur (JBP), Bandhavgarh (BDG), Devendranagar (DNG), and Raipur (RPR) than the rest of the provenances. The highest quantity of Rhein was obtained from the sample of Raipur provenance (31.94 µg/gm of dried leaf). The cluster analysis from the derived dendrogram depicted the presence of Rhein in higher amounts from the warmer provenances which corroborated with the study of Sakulpanich and Gritsanapan, (2009).

There are four phenological characteristics – Plant height, Number of pods, Number of flowers and three environmental factors - Altitude, Minimum temperature, Maximum temperature, and Rainfall. The relationship between Rhein quantities (µg/gm of dried leaf) with these seven phenological attributes was studied to identify the most important statistically significant predictor variable(s) with the help of Ordinary Least Square (OLS) regression. Table 8.1 showed the OLS statistics.

Table 8.1 Multiple R and R square values for phenological characteristics

Multiple R	0.895
R Square	0.801
Observations (# Provenances)	20

Multiple R denotes the correlation coefficient which signifies how good the data clusters around the fitted regression line. For a perfect linear fit the value of Multiple R should be 1.0. Here, the value of Multiple R is 0.895. This implies that the phenological characteristics and environmental factors are a very good fit of Rhein quantity (µg/gm of dried leaf).

R square denotes the percentage of variance explained by the Rhein quantity (µg/gm of dried leaf) can be captured by the predictor variables Number of pods, Number of flowers, Plant height, Altitude, Minimum temperature, Maximum temperature, and Rainfall with the help of the linear relationship. The value of R 0.801 denotes that more than 80% of the model variance is explained by the seven dependent variables. Table 8.2 showed the ANOVA results.

Table 8.2 Fitment of regression line for phenological characteristics

	df	SS	MS	F	Significance F
Regression	7	1724.271	246.325	6.892	0.002
Residual	12	428.872	35.739		
Total	19	2153.143			

From Table 8.2, the statistical significance is tested for the seven predictor variables as predictors of Rhein quantity (µg/gm of dried leaf). The F statistic value is 6.892, and the p-value is 0.002. This indicates that the regression equation fits the data well. Next, the regression equation and the significance of regression coefficients were studied. The results are shown in Table 8.3.

Table 8.3 Significance of regression coefficients for phenological characteristics

	Coefficients	Standard Error	t Stat	P-value
Intercept	-81.952	36.758	-2.230	0.046
Altitude	0.024	0.023	1.030	0.323
Min Temp	4.363	1.929	2.262	0.043
Max Temp	-1.635	1.449	-1.129	0.281
Rainfall	-0.011	0.007	-1.732	0.109
Plant Height	0.002	0.132	0.017	0.987
Number of Flowers	3.058	11.515	0.266	0.795
Number of Pods	-2.652	12.126	-0.219	0.831

Based on the values of the coefficients, the regression equation is constructed as follows:

Rhein quantity (μg/gm of dried leaf) = - 81.952 + 0.024 × Altitude + 4.363 × Minimum temperature - 1.635 × Maximum temperature - 0.011 × Rainfall + 0.002 × Plant height + 3.058 × Number of flowers - 2.652 × Number of pod.

From Table 8.3, it is observed that the t-statistics for Minimum temperature is statistically significant at the 0.05 level. This indicates that Minimum temperature contributes significantly to Rhein quantity (μg/gm of dried leaf).

The morphometric study of *S. obtusifolia* samples collected from twenty provenances was based on 22 important characteristics. Among these characteristics Plant height, Numbers of pods per plant, Pod length, and Number of flowers per plant had shown the maximum variability. The other three characteristics Root length, Internode length, and Rachis length showed a moderate level of variability. The remaining characteristics did not show much variability. LSD values obtained from these results confirmed these variations. Similar variations were observed by Jeruto et al., (2017) while studying 17 quantitative characteristics of *S. didymobotrya*.

The morphometric characteristics of *S. obtusifolia* were tested among themselves to know their inter-relationships. Some interesting inter-dynamics were observed. The Internode length appeared to be the most influential characteristic as it showed a highly positive correlation with Girth, Leaf length, Pedicel length, Leaf width, Sepal length, Anther length, Filament length, Pod length and Number of seeds per pod. The Filament length and Pod length were the next influential characteristics having a highly positive impact on eight other characteristics. Contrary to these, the two characteristics Root length and Number of branches were observed to be isolated from the others as they showed neither highly positive nor highly negative relationship with any other characteristics. The later one had shown a weakly negative correlation with the other morphological characteristics (Sonibare et al., 2004; Yousaf et al., 2010).

The relationship between Rhein quantities (µg/gm of dried leaf) with the considered morphological characteristics was studied to identify the most important statistically significant predictor variables with the help of OLS regression. Now, regression analysis cannot be performed with too many predictor variables. Therefore, the following important morphological characteristics identified by the PCA were considered for regression analysis:

Plant height, Internode length, Girth, Leaf length, Leaf width, Rachis length, Bract, Pedicel length, Sepal length, Filament length, Pistil length, Pod length, Seed per pod. Table 8.4 showed the OLS statistics.

Table 8.4 Multiple R and R square values for morphological characteristics

Multiple R	0. 959
R Square	0. 921
Observations (# Provenances)	20

The value of Multiple R is 0.959. This implies that the thirteen morphological characteristics are a very good fit of Rhein quantity (µg/gm of dried leaf) as a linear regression model. An R square value of 0.921 denotes that more than 92% of the model variance is explained by the fourteen predictor variables. Table 8.5 showed the ANOVA results.

Table 8.5 Fitment of regression equation for morphological characteristics

	df	SS	MS	F	Significance F
Regression	14	1982.088	141.578	5.348	0.025
Residual	6	171.055	28.509		
Total	20	2153.143			

From Table 8.5, the statistical significance is tested for predictor variables as predictors of Rhein quantity (µg/gm of dried leaf). The F statistic value is 5.348, and the p-value is 0.025. This indicates that the regression equation fits the data well. Next, the regression equation and the significance of regression coefficients were studied. The results are shown in Table 8.6.

Table 8.6 Significance of regression coefficients of morphological characteristics

	Coefficients	Standard Error	t Stat	P-value
Intercept	-100.601	48.512	-2.074	0.083
Plant height	0.534	0.203	2.631	0.039
Internode length	-165.381	151.128	-1.094	0.316
Girth	114.698	84.627	1.355	0.224
Leaf length	-406.980	350.334	-1.162	0.289
Leaf width	721.246	606.388	1.189	0.279
Bract length	-23.926	9.182	-2.606	0.040
Pedicel length	39.347	30.562	1.287	0.245
Sepal length	56.639	183.069	0.309	0.767
Filament length	-289.877	209.064	-1.387	0.215
Pistil length	-29.382	168.295	-0.175	0.867
Pod length	0.004	1.008	0.004	0.997
Seed per pod	60.708	48.075	1.263	0.254
Rachis length	20.913	18.575	1.126	0.303

Based on the values of the coefficients, the regression equation is constructed as follows:

Rhein quantity (µg/gm of dried leaf) = - 100.601 + 0.534 × Plant height - 165.381 × Internode length + 114.698 × Girth - 406.980 × Leaf length + 721.246 × Leaf width - 23.923 × Bract + 39.347 × Pedicel length + 56.639 × Sepal length - 289.877 × Filament length - 29.382 × Pistil length + 0.004 × Pod length + 60.708 × Seed per pod.

From Table 8.6, it is observed that the t-statistics for Plant height and Bract are statistically significant at the 0.05 level. This indicates that Plant height and Bract contributes significantly to Rhein quantity (µg/gm of dried leaf) in *S. obtusifolia*.

The PCA is a multivariate data analysis technique. It is useful for identifying principal components from a set of variables. Alternately, the technique helps in reducing the number of dimensions without losing much information represented by the original set of variables. Due to these reasons, PCA has become a popular technique of morphometric analysis which deals with several relevant characteristics related to different plant parts such as stem, leaves, roots, flowers, pod and seed.

For the *S. obtusifolia* samples, PCA was performed by considering the selected twenty-two characteristics for identifying major components. The results of the PCA revealed that the twenty-two characteristics can be mapped to four major components as there are only four eigenvalues which are more than one. The four major components with corresponding eigenvalues are shown in Table 8.7.

Table 8.7 Major components and eigenvalues of morphometric analysis

Component	Eigenvalue
1	15.560
2	1.671
3	1.472
4	1.245

In other words, the dimension of the morphometric space can be reduced from twenty-two to only four, and, hence, there are four major components for the morphological characteristics for the twenty *S. obtusifolia* samples from twenty provenances. It was also observed that two out of five general plant and leaf characteristics, two out of four fruit characteristics, and six out of eight flower characteristics loaded heavily to the first principal component. The result is shown in Table 8.8.

Table 8.8 Major significant characteristics associated with Rhein quantity

Attributes	Characteristics	Significant Characteristics
Plant	PLH, NBR, INL, GRT, RTL	INL, GRT
Leaf	LFL, LFW, RCL, STL, SPL	LFL, LFW
Flower	BRL, PCL, SLL, PTL, ANL, FML, PSL, NFL	BRL, PCL, SLL, ANL, FML, PSL
Fruit	NPD, PDL, NSP, SDW	PDL, NSP

Therefore, it can be concluded that characteristics related to flower are most important in determining the morphological properties of *S. obtusifolia* samples collected from various provenances.

This study is in accordance with the morphometric analyses were carried out by various researchers using PCA and cluster analyses (Soladoye et al., 2010a; 2010b; Jeruto et al., 2017). Intraspecific variations regarding various phenological and morphological characteristics among the other species of the genus *Senna* was studied by various researchers (Takuathung et al., 2012; Jeruto et al., 2017). In *Senna siamea,* different morphometric traits among nine provenances in Thailand related to growth and seed characters were recorded by Takuathung et al., (2012). Study of variations of morphological patterns among 39 populations of *S. didymobotrya* considering 17 qualitative and 17 quantitative traits were analysed by Jeruto et al., (2017) which corroborates with this study. Morphometric study concentrating on eight species of *Senna* considering thirteen morphological characters was carried out by Soladoye et al., (2010b). Interspecific morphometric variations among the different species of this same genus was observed by Rahman et al., (2013), where S. *sophera* is closely related to *S. hirsuta* in south-western Nigeria, while *S. sophera* and *S. multiglandulosa* were found to be allied closely in terms of morphological characteristics.

Population genetics considering morphological variations attracted attentions on genetic relatedness in several species of different genera (Gomez-Campo et al., 2001; Henderson and Ferreira, 2002; Sonibare, 2004; Bolourian and Pakravan, 2011) were also attempted in *S. obtusifolia*. Structural changes in the genome by mutations like deletion, translocation, inversion of a species that could separate them from their ancestors by attaining variations in morphological parameters is not an effective explanation (Tripathy and Goswami, 2011). The differences based on molecular markers and other genetic level studies could help analyse intraspecific genetic diversity.

The karyomorphological features studied for all the twenty accessions of *S. obtusifolia* showed the presence of 26 chromosomes in all the provenances with 4 chromosomes having NOR. The sum of the length of the longest and shortest arms represents the total length (HCL). Comparative study with the average data dealing with n = 13, the HCL varied from 27.19 µm for Asansol (ASN) provenance to 28.16 µm in Jabalpur (JBP) provenance. Figure 8.1 showed the graphical representation of the total genome length of all the twenty accessions of *S. obtusifolia.*

The minor increase in total Haploid chromosomal length indicates the gain of the chromatin material from different provenances. Variations in the whole length of chromosomes showing minor variations among all the accessions of *S. obtusifolia* may be due to the different ecological and edaphic factors and need more study for confirmation. Karyotype asymmetry analyses carried out according to the method of Peruzzi (Peruzzi and Eroğlu, 2013) of all the twenty accessions of *S. obtusifolia* revealed some minor variations among the provenances. Polyploidy were also observed in the sample of some of the provenances that corroborates with the studies of Resende et al. (2013) and Cordeiro et al. (2017).

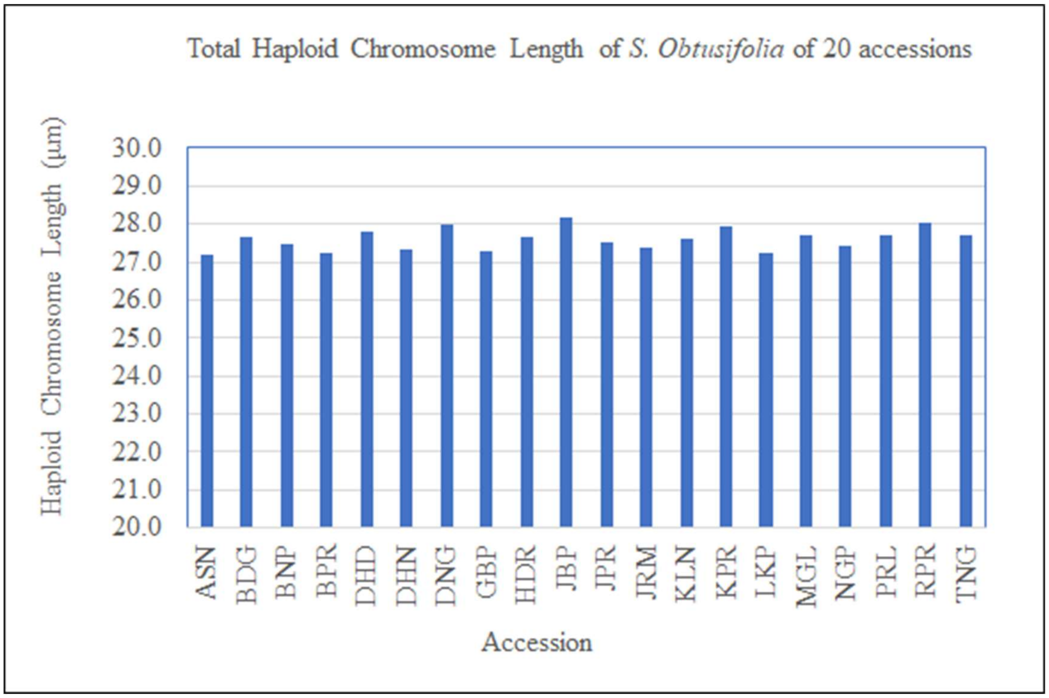

Figure 8.1 Total haploid chromosome length of *S. obtusifolia* of twenty accessions

Amplified fragment length polymorphism (AFLP) provided a deep insight in evaluating genetic diversity and phylogenetic relationships among the provenances. This technique of genome analysis is most modern, efficient, and robust with maximum reproducibility for determining infra-specific variation (Hodkinson et al., 2002; Archak et al., 2003). In this study, AFLP analyses were performed for the first time in *S. obtusifolia* species. A significant taxonomic subtle variation was observed among the provenances in the genetic characteristics. Out of the ten primer pairs combinations, the maximum amount of variation was found with the marker combination E-AAC/ M-CAG with 50% polymorphism but the overall polymorphism exhibited 33.11%. Total bands obtained were 453 out of which 150 were polymorphic. Thus, it may be concluded that the polymorphism observed may have some minor effects in the genome that might led to the phenological, morphological and biochemical variations and have also supported the meagre changes in the whole genome length in the karyotype asymmetry analyses among the species collected from different provenances.

The twenty samples of *S. obtusifolia* were analyzed and classified based on all the seven phenological characteristics, twenty-two morphological characteristics, 453 AFLP scores, and the Rhein quantity (µg/gm of dried leaf). The result of the cluster analysis is depicted in Figure 8.2.

Intraspecific diversity among twenty seed samples of *S. obtusifolia* were studied by Mao et al. (2017) regarding their secondary metabolites by HPLC and molecular study based on ISSR and SCoT markers revealed smaller variations.

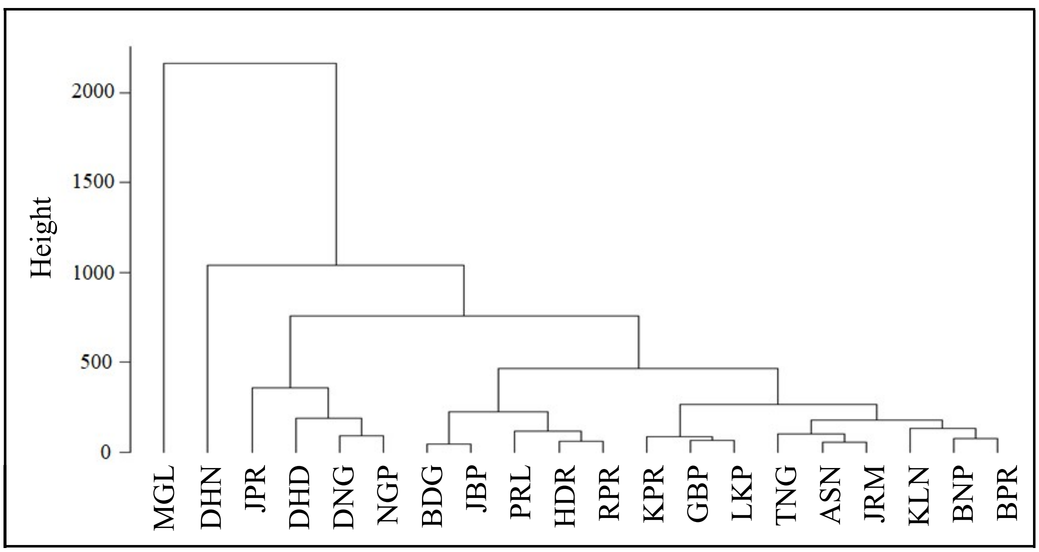

Figure 8.2 Overall dendrogram of phenetic analysis of 20 *S. obtusifolia* accessions

According to the dendrogram (Figure 8.2), the twenty *S. obtusifolia* samples can be grouped into five clusters. The samples from Mangalore (MGL) and Dehradun (DHN) are quite different from each other and from the rest as well. So, they belong to two separate clusters. The samples from Jaipur (JPR), Dahod (DHD), Devendranagar (DNG), and Nagpur (NGP) exhibit similarity with respect to the considered 482 characteristics, and hence belong to the third cluster. Out of these four samples, Devendranagar (DNG) and Nagpur (NGP) have least variations among themselves. Similarly, the samples from Bandhavgarh (BDG), Jabalpur (JBP), Purulia (PRL), Haridwar (HDR), and Raipur (RPR) exhibit similarity and belong to the fourth cluster. Within this cluster the pair of samples [Bandhavgarh (BDG), Jabalpur (JBP)] and [Haridwar (HDR), Raipur (RPR)] have minimum variations. Although, the sample from Purulia (PRL) belongs to this cluster, it has some distinctive feature compared to the two pairs. Finally, the samples from Kharagpur (KPR), Govindapur (GBP), Lakshmikantapur (LKP), Tatanagar (TNG), Asansol (ASN), Jhargram (JRM), Kalyani (KLN), Bishnupur (BNP) and Bolpur (BPR) exhibit minimum overall variations and belong to the fifth cluster. In this cluster, the the pair of samples [Govindapur (GBP), Lakshmikantapur (LKP)], [Asansol (ASN), Jhargram (JRM)], and [Bishnupur (BNP), Bolpur (BPR)] have minimum overall variations among themselves.

This cluster analysis establishes the relatedness of different extent among the twenty *S. obtusifolia* samples. Out of the twenty samples, ten were collected from the West Bengal and the remaining ten were collected from the other provenances of India. Interestingly, except the sample from Purulia (PRL), all nine provenances from the state of West Bengal belong to the same cluster. In other words, samples collected from more or less similar geographic and environmental conditions have shown similar phenological, morphological and genetic, and biochemical properties.

Tripathi and Goswami, (2011) reported that the variations in morphological characteristics can be influenced by the environmental factors and geographical locations. A spectrum of genes which may be responsible for the production of different biologically active molecules caused by various biological coordination finally constitute an organism forming an epigenetic system (Wagner, 1996). Changes in the production of any of these biomolecules at the lowest submicroscopic level can change in their macroscopic phenotypic level which may be addressed as an evolutionary change. In accordance, identifying such variations in static or dynamic states may act as a key factor for the conservation of the appropriate germplasm. In the process of evolution, this particular morphovarient will serve as the best germplasm for exploitation in the production of the active biomolecules.

In this study, collection of seeds or the germplasms of the species from the remote provenances all over the India and from most of the districts of West Bengal at the mature stage was quite challenging. Optimization of the Rhein extraction procedures from the leaf samples with several trials was also exciting.

These findings provide to serve the aid for further studies on the intraspecific forms together with their more detailed phenetic studies which may act as a good index to arrive at a decision regarding the identification of the best provenance of *S. obtusifolia* for the highest production of the bioactive molecule Rhein. Moreover, the presence of Rhein in other plant parts such as roots, seeds and stems of *S. obtusifolia* and their specific quantity with intraspecific variations from different provenances may be explored. A detailed and more specific study at the genetical level can throw light on the phylogenetic status of the species. Study of *S. obtusifolia* species under considerations of diverse array of provenances from various ecogeographical areas with varied ecological and environmental distribution may also be quite interesting for identifying the zones containing the maximum Rhein quantity in the respective samples.

In drawing general inference of this entire study, it may be commented that *S. obtusifolia* collected from twenty different provenances showed variations on different phenetic characters that have been analysed thoroughly and independently throwing light in the biochemical and genetic levels. Moreover, natural consideration of the species with greater heights and longer bracts growing mostly in warmer zones are the best producer of Rhein in comparison to the others.