**CHAPTER 6**

**SUMMARY AND CONCLUSIONS**

*Tinospora cordifolia*, a climbing shrub belonging to the family Menispermaceae is widely distributed throughout Indian subcontinent and China. Aqueous extract of stem and root of the plant has been used therapeutically because of immunomodulation property as well as antimalarial and antileprotic activities. The aqueous extract contains a number of chemical constituents including alkaloids, steroids, glycosides, polysaccharides. The practice in Ayurveda: Indian System of Medicine is to prescribe decoction of stem of *Tinospora* with *Piper longam* in malarial fever. It helps in reducing splenomegaly as well. The alcoholic extract of the plant is prescribed in Ayurveda and Allopathy as an immune promotor. *T. cordifolia*, are good immunity-promoting drugs which can play an important role in primary health care for the prevention, promotive and curative points of view. *T. cordifolia* has become an imperative subject of research due to the presence of numerous pharmaceutical valuable bioactive compounds. In our modern era, impressive research has been done on the biological activity and possible application of Guduchi and its chemical constituents. Guduchi may have been favorably used for thousands of years but modern herbal pharmacology appears to have just begun to appreciate “The One Who Protects the Body”.

*Tinospora cordifolia* is an important herb of India and holds a special position as a potent adaptogen in Ayurvedic System of Medicine. Conventional propagation methods such as stem cuttings, though useful for propagation, are dependent upon weather conditions for proper growth. Secondly it suffers from poor seed set and poor germination which led to its scarcity in natural habitat. Keeping this in view, the present investigation was undertaken with the aim to characterize the collected germplasm for identification of elite genotypes based on bio-active constituents. Study also investigated the question concerning the extent of genetic diversity existing among *Tinospora* accessions. Wild and cultivated samples from eleven Indian states along with cultivated samples from National Bureau of Plant Genetic Resources (NBPGPR, New Delhi) were collected during survey and collection of giloy (September 2010 to March 2011) for analyzing the extent of genetic variability. Many nurseries were also visited during the explorations. During survey involvement of local and tribal communities played a key role in sample collection as well as under studies its applications for human welfare.
Ten Cuttings (hardwood) of each genotype (total 60) were established in the Green house of Centre for Plant Biotechnology, CCS HAU Campus, Hisar using stem cutting method with the treatment of auxins (IBA, NAA). Many of these accessions have been transferred to the field.

Biochemical analysis of 60 accessions from different eco-geographical locations displayed highest concentration of Berberine (0.132 µg mg⁻¹) in the sample collected from Indian Institute of Integrative Medicine (TC-2, IIIMJ-2), (Jammu, Jammu & Kashmir), whereas the giloy plants gathered from Ambala, Haryana (TC-20, HRAB) exhibit least concentration of Berberine (0.014 µg mg⁻¹). Through HPLC analysis of stem samples of *Tinospora cordifolia* a valid quantitative range of Berberine (0.019 µg mg⁻¹ to 0.132 µg mg⁻¹) was found while analyzing 60 accessions for estimation of alkaloids through reverse phase HPLC. A broad disparity was observed regarding Berberine concentration among all collected accessions. The concentration of production of secondry metabolites is likely to be influenced by various geographical (different rainfall and temperature) and growth (developmental stage) factors. Further identification of high Berberine producing lines will play an important role in designing mass propagation as well as conservation strategies in future. These superior genotypes can also be used in forward genetics (molecular characterization of gene(s) corresponding to Berberine production) and its isolation and integration in other plant or micro-organisms for production of Berberine.

For successful breeding programs appropriate recognition of source of planting material is of prime importance, which requires a reliable and authentic system of characterization. Out of total 50 RAPD and 50 ISSR primers 44 and 36 primers produce reproducible bands, respectively. Analysis of RAPD, ISSR and combined data of RAPD and ISSR produce a significant polymorphism with 64.81%, 63.15% and 63.98%, respectively. Highest degree of polymorphic bands per assays was obtained in RAPD (7.15) as compared to ISSR (4.30) or combined analysis of RAPD and ISSR (5.72). Size of amplification products ranged from 150-2000 bp in RAPD, while 200-2200 bp in ISSR. RAPD analysis revealed higher diversity index, marker index and resolving power values (0.87, 4.41 and 12.57), but a lower PIC (0.14) then ISSR or RAPD and ISSR data combinely. The UPGMA dendrogram revealed clustering of different accessions into four major groups based on similarity value. In RAPD dendrogram, The cluster I was further divided into 2 sub clusters with 2 (TC-18 and TC-27) and 6 accessions respectively. In case of ISSR, The cluster I was
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further divided into 2 sub clusters with TC-27 (IC-550229) and 6 accessions. II\textsuperscript{nd} Major Cluster contains 13 accessions while III\textsuperscript{rd} enclosed 27 accessions separated into two sub–clusters and IV\textsuperscript{th} cluster contained 13 accessions. Combined data of RAPD and ISSR also generated dendrogram showing four major clusters and further divided into two subclusters each and with 8, 11, 26 and 15 accessions respectively Genetic similarity calculated from scoring data of RAPD, ISSR and RAPD+ISSR markers using Jaccard’s coefficient among 60 accessions of \textit{Tinospora cordifolia} collected from different locations of four eco-geographical regions of India revealed high genetic similarity values.

The Relevance and Scope of the Proposed Study

\textit{Tinospora cordifolia} is one of the threatened species which is becoming rare day by day due to human impact on forests including overexploitation and increasing biotic and abiotic stress. Berberine is the chief alkaloid in \textit{T. cordifolia} extracts has been found to exert beneficial effects on the cardiovascular system and significant anti-inflammatory activities. No advance cultivars are available or selected in this important medicinal plant due to the lack of characterization and evaluation studies. Genetic variability of the species is under threat due to low dispersal of seeds and clonal reproduction. In this context the proposed study will provide worthy assemblage of \textit{Tinospora} germplasm from diverse location throughout the country with the knowledge of higher Berberine producing elite lines. The study also imparts worthy genetic information including classification of elite lines of giloy germplasm with reference to Berberine content and characterization of genetic diversity will help in devising appropriate breeding strategies. Higher Berberine containing accessions identified which will provide important insight as to which kind of germplasm should be multiplied in mass. Statistical analysis proves that the quantification procedures are repeatable and selective for the estimation of this alkaloid and can be used in standardization purposes and pharmacokinetics. This course of action can be used in detection of the related impurities in therapeutic formulations and stability of drugs in industries. All the studies found to be relevant in the present day position when the species is heading towards extinction; the available germplasm is not well characterized. The outcome of the study will be very helpful in initiating a number of crop improvement ventures on this plant species. The work can be helpful for pharmaceutical industries for the development of new drugs for disease like cancer and hypertension.
Future Directions

Despite high market demand of highly valuable bioactive constitutes in *Tinospora* species significant efforts have been made for large scale cultivation in farm lands or in orchards. Therefore, it is necessary to support the local people and tribal communities for large scale cultivation of this promising medicinal tree. This species is found as natural wild and is propagated mainly through cuttings so no commercial cultivars are available. Chemical profiles through highly sophisticated analytical studies can be used for extensive exploration of phytoconstituent pockets of this medicinal dosage form. This may bring Physicians nearer in achieving specific anticipated desirable action by clearing them what season to opt for drug collection and preparation of *satva*.

Further conservation of available genetic variability is essentially required for selection of desired genotypes for use while formulating and implementing reforestation and plantation programs. For the conservation of this species, rapid multiplication and rehabilitation in its natural habitat is necessary. To overcome this threat, a reliable method of quick multiplication like tissue culture and methods of *in-situ* as well as *ex-situ* conservation could well provide a viable solution to the problem.

**CONCLUSIONS**

- A total of 60 accessions were collected from different eco-geographical regions of eleven states and one National Capital region of New Delhi.
- Collected accessions were established in the Green house condition using root cutting in polybags containing a mixture of sand and manure (3:1 ratio) and used for optimization of biochemical and molecular marker analysis.
- An efficient protocol for Berberine extraction from stem samples was developed for characterization of the collected germplasm through HPLC.
- In HPLC analysis among different geographical locations highest concentration of Berberine (0.132 μg mg\(^{-1}\)) was found in the samples collected from Indian Institute of Integrative Medicine (TC-2, IIMJ-2), (Jammu, Jammu & Kashmir). Whereas least concentration of Berberine (0.014 μg mg\(^{-1}\)) was found in wild accessions (TC-20, HRAB) collected from Ambala, Haryana.
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- Genetic characterization among 60 accessions of *Tinospora* through RAPD revealed significant genetic variability with 64.81%. 44 primers out of 50 were found to be polymorphic with 315 amplified bands. All accessions grouped into four major clusters in which cluster I, III and IV further divided into two sub clusters. Polymorphic loci varied from 58.38% to 90.36% through POPGENE analysis via grouping all 60 genotypes of *T. cordifolia*.

- 63.15% polymorphism was obtained in ISSR analysis with an average of 4.30 polymorphic bands per assay in 36 primers out of 50. Four clusters contained 7, 13, 27 and 13 accessions respectively in each cluster. POPGENE analysis revealed highest value of Heterogenity in Himalayan region (0.306), whereas lowest in Coastal and Arid region (0.229).

- Combined analysis of RAPD+ISSR revealed 63.98% polymorphism with 471 polymorphic and 265 monomorphic bands. The percentage of polymorphism varied from 59.35% to 91.43% (highest in Gangetic region, while lowest in Coastal region).

- The genetic similarity coefficient among 60 accessions of *Tinospora* ranged from 0.58-0.92.

- This study aimed not only to identify high Berberine producing lines, but also useful in assessing the influence of environmental characteristics on the production of secondary metabolites, as well as evaluating the genetic variability in the collected accessions.

- AMOVA among 60 genotypes of 4 different eco-geographical regions revealed 22% variation among the group and 77% variation among cultivars. With respect to RAPD marker berberine content gene flow is 2.114 while with respect to ISSR it is 2.279 and on the basis of the combined data of RAPD and ISSR gene flow is 2.176.

- To the best of my knowledge, this is the first report about biochemical and molecular characterization of wide range collected accessions of *Tinospora cordifolia* from different eco-geographical regions throughout the country.