Chapter 1
INTRODUCTION

India has been referred to as a mega-diversity nation on account of its enriched faunal and floral variations. It encloses a range of biodiversity hotspots among which the Himalaya Hotspot harbours several indigenous and endemic species. The Eastern Himalayan region comprises of the Darjeeling hills (average 6700ft) sharing borders with Bhutan and Sikkim in the North East and Eastern Nepal in the west being therefore deemed to be a characteristic geographic terrain that unifies the diversified flora of Bhutan, India and Nepal.

Climbing species play a critical role in intensifying the floristic richness of this naturally scenic landscape amidst the magnificent Tea gardens. The Gourd family Cucurbitaceae holds the second position after Papilionaceae in respect of the climber dominating angiospermic families of Darjeeling hills with around twenty seven reported species. Among the collective 11.61% endemic climbers persisting in Darjeeling region of Eastern Himalaya (Samanta, 1998); an under explored ethnomedicinally relevant indigenous climbing Cucurbit is *Herpetospermum darjeelingense* (C.B.Clarke) H.Schaef. & S.S.Renner [*Edgaria darjeelingensis* C.B.Clarke]. The plant has been stated to be endemic along with being previously reported as an endangered floral entity (Samanta, 1998; Chakraborty *et al*., 2021a).

The Darjeeling hills are located between 27.0410°N and 88.2663°E geographical coordinates with the indigenous Cucurbit being sheltered at altitudinal range of 1450-3000m. *Herpetospermum darjeelingense* is a typical shrubby climber characterized by axillary shoot tendrils employed for climbing purposes. Morphologically, the observable tendrils in the plant are modified axillary buds specialized in providing support and anchorage to the vining stem. Traditionally *Herpetospermum darjeelingense* is

known as ‘Cathil’ in the Chitwan district of Nepal with its fruits being economically valued as a source of vegetable by the local people comprising representative members of the ancient tribal ethnic group ‘Tharu’ (Mueller-Boeker, 1993). The ‘Tamang’ community in centralized locations of Nepal and Darjeeling Eastern Himalaya also has been reported to use the climbing cucurbit plant in herbal veterinary formulations being commonly referred to as ‘Tangsarkato’ and ‘Jangali Karela’ by the Tamang’s and local Nepali inhabitants respectively. The seeds of *Herpetospermum darjeelingense* have been stated to be used in bovine treatment as per the ethno-medicinal based traditional knowledge of the ‘Tamang’ community. The treatmental methodology involves the mixing of the pulverized plant seeds with corn flour being subsequently fed to the cattle’s in order to relieve them from bovine pyrexia (Shrestha and Khadgi, 2019). A solitary report by Chakraborty *et al*. (2021a) too highlighted the potential biomolecules conferring remarkable antioxidant property to the cucurbit climber with insights on its future pharmaceutical aspects.

Other than these few traditionally available ethnic and documented data on *Herpetospermum darjeelingense*, the plant has remained completely neglected in respect of scientific investigations in multiple dimensions. There are no reports on its molecular systematics analysis including a missing in-depth understanding of the metabolomic composition and *in vitro* phytochemical characterization of the plant. Besides conservation strategies through *in vitro* regeneration technique owing to its endemic status is also an essential perspective to be looked at. Climate change, habitat destruction due to urbanization (Chhetri *et al*., 2005) and expansion of commercial tea plantations are considered as probable reasons for restricted distribution and imposition of endangered status to numerous plants of the Himalaya hotspot. However, the possibility of disease infestation and its consequential detrimental effects in the diminishing of these susceptible floral specimen’s viz. *Herpetospermum darjeelingense* has been completely overlooked to the fact that most endemic plants of this region has not been delineated based on its endophytic traits. The present

work aims to establish a molecular identity of the plant along with probing and unraveling the biomolecular attributes of the unexplored Cucurbit climber besides exerting a special emphasis on its tissue culture based regeneration aspects towards natural preservation of the floral specimen. Further analyzing the impact of tea plantations on the existence of *Herpetospermum darjeelingense* is of utmost importance in order to understand the influence of ecological impact in its distribution pattern including a vivid understanding of the endophytic features of the plant.

In accordance with the Modern angiosperm phylogeny group IV (APG IV) system of taxonomic classification, order Cucurbitales is placed in the clade Rosids, superclade Superrosids; which is hierarchically placed within the clade Eudicots of Angiosperms clade respectively. Cucurbitales Juss. ex Bercht. & J.Presl order comprises the families Apodanthaceae Tiegh. ex Takht.; Anisophylleaceae Ridl.; Corynocarpaceae Engl., *nom. cons.*; Coriariaceae DC., *nom. cons.*; Cucurbitaceae Juss., *nom. cons.*; Tetramelaceae Airy Shaw; Datiscaceae Dumort., *nom. cons.* and Begoniaceae C.Agardh, *nom. cons.* A comprehensive study of phylogenetic relationship in the order Cucurbitales employing fourteen DNA loci from three organellar genomes, specifically the plastidal *rbcL*, *matK*, *ndhF*, *atpB*, *trnL*, *trnL-trnF*, *rpl20-rps12*, *trnS-trnG* and *trnH-psbA* genes including introns and spacers; the mitochondrial *nad1 b/c* intron and *matR* gene; in addition to the nuclear ribosomal genes 18S, ITS1-5.8S-ITS2 and 28S was conducted (Schaefer and Renner, 2011a). The overall dataset comprised of 664 in-group species, featuring all apart from two genera corresponding to about 25% of the total two thousand six hundred species in the order Cucurbitales. Maximum likelihood type phylogenetic tree analysis produced almost matching topologies in respect of the cumulative molecular datasets retrieved from the three respective organellar genomes imparting associative relationship information among the eight families under order Cucurbitales in the sequential order of (Apodanthaceae, Anisophylleaceae, (Cucurbitaceae, ((Coriariaceae, Corynocarpaceae), (Tetramelaceae, (Datiscaceae, Begoniaceae))))) (Schaefer and

Renner, 2011a). Depending on the foundation of molecular characterization including review of literature based morphological data scrutiny, the recircumscribed tribes and genera under the family Cucurbitaceae was presented, which portrayed a more naturally reliable and informative classification mode in respect of this angiospermic family. This novel system constituted a summative of 95 genera placed under 15 analogous tribes with five of them being entirely newly introduced viz. Actinostemmateae, Indofevilleeae, Thladiantheae, Momordiceae and Siraitieae (Schaefer and Renner, 2011a). The schematic arrangement required forty four new combinations as a step towards formal nomenclature with inclusion of two new names in the family Cucurbitaceae (Schaefer and Renner, 2011a).

Edgaria darjeelingense C.B.Clarke was transferred to the genus *Herpetospermum* on the basis of cumulative morphological and molecular evidences being renamed to *Herpetospermum darjeelingense* (C.B.Clarke) H.Schaef. & S.S. Renner (Schaefer and Renner, 2011a). The genus *Herpetospermum* was positioned in Tribe No. 11 named Schizopeponeae C. Jeffrey in Kew Bull. 17:475. 1964. Presently, the genus *Herpetospermum* is comprised of the following three species namely *Herpetospermum darjeelingense* (C.B. Clarke) H. Schaef. & S. S. Renner, *Taxon*, 60(1): 134. 2011 (genomic number n=11); *Herpetospermum pedunculosum* (Ser.) Baill. *Hist. Pl.* 8:445. 1885 and *Herpetospermum tonglense* (C.B. Clarke) H. Schaef. & S. S. Renner, *Taxon*, 60(2):615 (2011c) being distributed on riverbanks in and around India, Myanmar, Nepal, Tibet and China (Thakur and Sinha, 1973; Schaefer and Renner, 2011a&b).

Very sparse data on biomolecular attributes and usages have been reported among the tribal representative of Schizopeponeae. Most of the research work and information have been limited to *Herpetospermum pedunculosum* C.B.Clarke (synonymous to *Herpetospermum caudigerum* Wall.). The plant is extensively recognizable in Tibetan medicine where its seed having a bitter taste has been characterized to show cooling potency. Anti-inflammatory activity against stomach and intestine, choleretic, cholagogue with febrifuge

potential including being used in treatment of troubles related to hepatic problems and bile duct associated issues have also been delineated by earlier workers employing *Herpetospermum pedunculosum* extracts (http://www.flowersofindia.net/catalog/slides/Himalayan%20Bitter%20Gourd.html). Three biologically active lignan class compounds have been isolated in pure form from *Herpetospermum caudigerum* seeds namely Herpetone, Herpetin and Herpetfluorenone; characterized to demonstrate anti-hepatitis B viral activity (Gong *et al*., 2017) in addition to displaying confirmed hepatoprotective effects on Carbon tetrachloride induced hepatic fibrosis disorder in rats (Feng *et al*., 2018). Five other novel compounds deduced in ethyl acetate extracts of *Herpetospermum caudigerum* seeds have also been reported viz. 2-propenyl ester, Cucurbitacin R, Cucurbitacin L, 3'-Hydroxydaidzein and Oleanic acid featuring unique biochemical attributes (Dai *et al*., 2017).

Thus, it is observed that no investigations on phytochemical and molecular biological characterization of *Herpetospermum darjeelingense* have been reported till date with quite an amount of work been accomplished based on its sister species *Herpetospermum pedunculosum*, being affirmed to possess high medicinal value. Isolation of biomolecules in pure form and its biosynthetic mechanisms is also yet to be studied in these genera. Moreover, preservation of this endemic plant stressed by anthropogenic and ecological factors requires employment of *in vitro* conservation methodology. Among the indigenous cucurbit of Darjeeling hills *Herpetospermum darjeelingense* was a neglected name. Other than the study of taxonomic features and distribution of the plant in the hilly geographic terrain, no significant work on the plant has been previously done. Morphology based taxonomic identification was the only mode possible for accurate identification of the species. The research work aimed to fill in the information gap through a comprehensive study of all major parameters related to the plant along with linking and validation of the ethnomedicinal based available traditional knowledge to the actual biomolecules behind displaying such noble medicinal values. To fill in this

research gap, the present work was designed with the following objectives-

1. Collection and authentication of plant material through DNA barcoding and its bioinformatic analysis- Morphology based taxonomy was the only methodology available for identification of the plant. Molecular data based authentication and further bioinformatic analysis owing to solving the phylogenetic puzzle can help to unravel the evolutionary traits of *Herpetospermum darjeelingense*.
2. Study of ecological status of the plant through sampling of metrological data; pedological analysis via evaluation of soil pH, moisture content, electrical conductivity, organic carbon, organic matter including evaluation of total Nitrogen, available Phosphorous, Potassium, Sulphur and soil micronutrient status- Investigation on ecological status would provide a fair understanding of the environmental factors essential for survival of *Herpetospermum darjeelingense*.
3. Qualitative analysis of sample through study of foliar pH, moisture content, crude fibre, and ash value including study of overall biochemical attributes of the plant with respect to occurrence of flavonoid, phenol, cardiac glycoside, coumarin, steroid, tannin and terpenoid- Study on qualitative features would decipher a clear idea of the nature of biochemical constituents occurring in *Herpetospermum darjeelingense*.
4. Study of *in vitro* antioxidant and anti microbial activity- *In vitro* methodology of quantitative evaluation of a range of non-polar to polar solvent based sample extracts of the plant under the following subheads would help to understand the antioxidant potential of the plant with a typical hint on the nature of the antiradical compounds in the sample concentrates. Further chromatographic separation based 2,2-diphenyl-1-picrylhydrazyl (DPPH) analysis will help to reveal the best solvent responsible for solubilizing the bioactive compounds in *Herpetospermum darjeelingense*.
    - *In vitro* technique of DPPH scavenging potential.
    - Ferric reducing power (FRP) assay.
    - Total flavonoid content analysis.
    - Column-chromatographic separation.
    - Detection of phenolic content.

- Hydrogen-peroxide quenching activity.
- Nitric oxide test.
- Assessment of antimicrobial efficacy of plant extracts.

4. Synthesis and study of biogenic Silver nano particles- *Herpetospermum darjeelingense* biogenic extract based Silver nano particles (AgNP's) synthesis and characterization can help to determine the extent to which antimicrobial, antioxidant and anti-lipid peroxidation efficacy is increased on account of nano particle production with its potential in pharmaceutical implications.
5. Study of Gas Chromatography-Mass Spectrometry (GC-MS) detected metabolites- GC-MS analysis can help to reveal the metabolic biomolecules being synthesized in *Herpetospermum darjeelingense* along with providing a comprehensive data on biomolecular profiling aspects.
6. Identification of GC-MS detected bioactive molecules- The identification of bioactive molecules in *Herpetospermum darjeelingense* extract can help to trace which compounds are responsible for providing ethnomedicinal values to the plant.
7. Evaluation of metabolomic variation in plants growing in tea and non-tea growing regions of Darjeeling hills with a hint on ecological stress impact- This methodology would help to understand the impact of tea gardens in altering the metabolomic composition of *Herpetospermum darjeelingense* with a hint towards how ecological stress factors play a role in influencing the biosynthesis of metabolomic entities.
9. Study of chemotaxonomic variation among plants growing in manifold locations through binary scoring technique towards assessment of relatedness with dendrogram generation- The binary scoring and correlation study would help to decipher the biochemical relatedness among *Herpetospermum darjeelingense* samples growing in varied ecological locations.
10. Study of metabolomics of bioactive molecules, highlighting the cardinal genes controlling the pathway- Metabolomics would help to understand how these varied biomolecules are synthesized in the plant with further prospects in metabolomic bioengineering in relation to key genes controlling the enzymatic machinery behind metabologenesis of the detected compounds.

11. Study of metabolomic bioenergetics- Study of metabolomic bioenergetics can help to understand the energy cost of metabolic entities and to understand whether there is excess waste of metabolic energy towards synthesis of bioactive molecules.
12. Study of *in silico* and *in vitro* hepatoprotective activity of some detected molecules- The hepatoprotective attributes of the sample extracts can be analyzed along with molecular docking study of the identified hepatoprotective compounds through GC-MS against liver disorder associated protein targets indicating the potential of these compounds as anti-lipid peroxidation agents.
13. Study of *in silico* and *in vitro* antidiabetic activity of some detected molecules- The antidiabetic potential of the sample extracts can be analyzed along with molecular docking study of the identified antidiabetic compounds via GC-MS against diabetes related proteins indicating the efficacy of these compounds as anti-diabetic drug targets.
14. Isolation of pure compounds- Pure compounds can help to validate the individual contribution of potential molecules in an extract against various ailments through *in vitro* analytical methodology with an option of drug development.
15. *In silico* analysis of isolated compounds in the form of physicochemical characterization, bioactivity score calculation including absorption, digestion, metabolism, excretion and toxicity (ADMET) analysis- Bioinformatics based investigation can help to compare the physicochemical characters of the isolated pure compounds to established drug molecules along with providing insights on ADMET features in order to understand the pharmacokinetic properties of test drug compounds.
16. Molecular docking of isolated compounds against proteins associated with cancer towards studying the efficacy of the ligands in treating those diseases- Before proceeding to *in vitro* experiments, molecular docking study can help to decipher the potential ability of the isolated molecules as anticancer agents through binding efficiency of the drug (ligand) to the cancer related proteins.
17. *In vitro* assay of isolated pure compounds against cancer cell lines associated with breast and lung cancer- After *in silico* analysis the

pure compounds are tested against breast and lung cancer associated cell lines in order to estimate their efficacy in inhibiting the growth of the cancerous cells through *in vitro* methodology.

18. *In vitro* regeneration and hardening of *Herpetospermum darjeelingense* plants- Tissue cultured based propagation can aid in conservation of this indigenous floral entity.
19. Comparative metabolomics of naturally originating and *in vitro* regenerated plants- The comparative metabolomic analysis of naturally growing and tissue cultured *Herpetospermum darjeelingense* plant samples can help to understand the alteration in biomolecular entities in the corresponding specimens as investigated through GC-MS analysis.
20. Understanding the endophytic traits of the plant- To investigate whether there is any endophyte residing within the climber plant with an aim to explore and unveil the influence of biotic stress imposed by the concealed endophyte if any through antagonization of biologically active and essential secondary metabolites in *Herpetospermum darjeelingense*.