ABSTRACT

The concept of biodiversity hotspot is an effective methodology to identify various potential regions around the globe where immediate attention is required for addressing the alarming issues of loss in floral and faunal biological diversity inclusive of framing standard operating procedure towards designing conservation strategies. India is renowned as a mega-diversity nation on account of its rich floral and faunal variations. It harbours several biodiversity hotspots of which the Himalaya Hotspot serves as a habitat to numerous endemic and indigenous species. The Eastern Himalayan region inclusive of the Darjeeling hills possessing average altitude of 6700ft shares boundaries with Eastern Nepal in the west along with Bhutan and Sikkim in the North East. Therefore, this area can be considered as a typical geographic terrain which assemblages the diversified endemic-cum-exotic flora of India, Bhutan and Nepal.

Darjeeling Himalaya is well known for its diverse and richest range of vegetation in India. Its rich flora and fauna is of paramount significance for the nature lovers and biologists serving as one of the richest and interesting botanical regions in the whole of Indian subcontinent and thus, has been a central point of natural and floristic attraction for tourists and nature lovers. Increase in population of the district, urbanization, construction of roads, dams and power projects, deforestation, clearing of forests for cultivation, construction of resorts for eco-tourism, forest fire etc. can be ascertained to be the prime reasons behind the rapid decline in biodiversity of this area which are facing a tremendous threat to the extent of their existence. Tremendous biotic pressure, mostly anthropogenic, directly or indirectly on these forests is causing serious problems to the very survival of numerous species of plants and animals. Large scale deforestation also took place for extracting timber. Deforestation has resulted in fragmentation of forests and habitat destruction, thus posing a great threat to the regional biodiversity. Introduction, escape and naturalization of exotic species are also exerting tremendous pressure on the local vegetational structure affecting the survivability of numerous indigenous species. Officers of the British government in India sometimes during the early twentieth century spread the seeds of *Cryptomeria japonica* on different sites of Darjeeling hills, which in course of time, has modified the forest structure. The high resin content of these naturally rejected plant parts take a long time to decompose and gradually made the habitat unsuitable for the growth of other plant species. Practice of monoculture of broad leaved tree species such as *Tectona grandis*, *Exbucklandia populnea*, etc. besides conifers such as *Cryptomeria japonica*, *Pinus* spp. and so forth has lead to destruction of natural habitat in wide areas affecting the overall composition of Darjeeling flora through length and breadth. However, documentation of the comprehensive natural resources is still far from complete. One of the key reasons behind this is the inaccessibility factor in ground based survey of many far flung

areas compounded with terrain complexity.

Darjeeling tea akin to Scotch whisky is a specialized industry. The bouquet and aroma of this brew cannot be replicated. The tea plantations have had an adverse impact on geo-ecological and socio-economic conditions of people in the Darjeeling Hills. The increase of tea plantations with just one tea estate in 1856, thirty nine in 1866, one hundred thirteen in 1874 and one hundred eighty six by 1905 seems to have led to large-scale deforestation, landslides, soil erosion, loss of wildlife and biodiversity. At the present time, the number of tea estates has reduced to about eighty seven but the quantity of plantation land has remained constant, exerting additional pressure on the local ecology and economy. Moreover, the use of pesticides, herbicides and fertilizers has caused contamination of rivers-cum-streams leading to degradation of soil. The rugged and largely inaccessible, landscape makes biological surveys in the eastern Himalayas extremely difficult. Immense areas of intact forests are either slightly or entirely unexplored. Therefore, numerous floral and faunal taxonomic groups are understudied and the factual extent of the biological diversity is indisputably underestimated. But, despite the scant knowledge, it can be affirmed on the basis of what we know that the eastern Himalayan region is amongst the biologically richest areas on the planet Earth with augmentation of tea plantations in Darjeeling being a real threat to floral diversity.

India has been a potential hub of traditional herbal medicine since the Vedic ages. Numerous works have been conducted in documentation of traditional knowledge and exploration of ethnomedicinally important floral entities. Despite several investigations, some medicinal plants owing to their indigenous and endemic status have been overlooked. Out of the various aboriginal species and among the 11.61% indigenous climbers of the Darjeeling Eastern Himalayas; an ethnomedicinally significant but phytochemically under investigated endemic Cucurbit climber that has remained neglected from the advantages of molecular identity besides missing investigations on any kind of biochemical and metabolomic characterizations till date is *Herpetospermum darjeelingense* (C.B.Clarke) H.Schaef. & S.S.Renner [*Edgaria darjeelingensis* C.B.Clarke]. The plant is not only endemic but was also enlisted under the endangered category confronting a very high risk of extinction in the wild in the near future as per scientific records besides facing immense ecological threats due to unplanned expansion of commercial tea gardens in Darjeeling hills. The angiospermic family Cucurbitaceae ranks second (only next to Papilionaceae) among the climber dominating families in the Darjeeling hills of Eastern Himalaya with about twenty seven reported species. The referred plant occurs at an altitude of 1450-3000m and is a climber characterized by axillary shoot tendril with axillary buds being modified into tendrils for climbing purposes. *H. darjeelingense* is prevalently known as Cathil in the Chitwan district of Nepal and its fruits are economically useful as a source of vegetable to the local people specifically in regard to the ancient tribal ethnic group ‘Tharu’. The ‘Tamang’ community in Central Nepal and Darjeeling Eastern Himalayas also

employs *H. darjeelingense* in herbal veterinary medications where it is commonly known as 'Tangsarkato' among the Tamang's and 'Jangali Karela' among the local Nepali inhabitants. The seeds of the plant are mentioned to be used for bovine treatment as per the ethno medicinal records of the 'Tamang' community. The methodology involves the mixing of the pounded seeds with corn flour being fed to the cattle for relief from fever.

Therefore, on account of unavailability of any research data other than its reported usage as a vegetable and veterinary medicine; the present research work focused to fill in the information gap related to *Herpetospermum darjeelingense* through DNA barcoding; ecological analysis; qualitative and quantitative biochemical tests; biogenic synthesis of Silver nano particles; GC-MS probe; comparative metabolomic study of *H. darjeelingense* plants distinctly growing in tea and non-tea growing areas; chemo-taxonomic variation analysis; study on metabologenesis and bioenergetics of the bioactive compounds with investigation on cardinal genes; *in silico* and *in vitro* investigation of hepatoprotective and antidiabetic activity of plant extracts; isolation of pure compounds; physicochemical characterization, ADMET (Chemical absorption, distribution, metabolism, excretion and toxicity) and molecular docking based study of isolated compounds; *in vitro* anti-cancer assay; employment of tissue culture technique as a step towards *ex situ* conservation of the plant besides studying the endophytic traits of the plant.

Other than morphological feature based taxonomic identification, no other modern approaches for recognition of *Herpetospermum darjeelingense* was available. Therefore, molecular data in respect of this plant can help in solving phylogenetic puzzles of the Cucurbitaceae family with a portrayal of its underlying rbcL DNA based signature aiding in correct identification of the plant. Since, the plant has been depicted to possess ethnomedicinal implications in veterinary medication; the present study would decipher biomolecular signatures responsible for imparting medicinal properties to the plant validating the available traditional knowledge of this plant. Isolation of bioactive molecules from this plant can enhance the information pool and help in treatment of various complex ailments. Metabolomic analysis of GC-MS detected metabolites can pave way for further research on metabolic bioengineering. Ecological threat and exploitation of plant for isolating potential bioactive molecules can further deteriorate its restricted distribution; thereby *ex situ* conservation measures through rapid multiplication via *in vitro* regeneration techniques could be a boon to both pharmaceutical industry and biodiversity conservation strategies.

Modern angiosperm phylogeny group IV (APG IV) system of classification places order Cucurbitales in the clade Rosids, superclade Superrosids; being successively placed within the clade Eudicots of Angiosperms clade respectively. The order Cucurbitales includes the families Apodanthaceae, Anisophylleaceae, Corynocarpaceae, Coriariaceae, Cucurbitaceae, Tetramelaceae, Datiscaceae and Begoniaceae. Inquiry on phylogenetic association of the order Cucurbitales was

conducted employing fourteen DNA loci from three organellar genomes, specifically the plastidal, mitochondrial and nuclear ribosomal genes. Maximum likelihood based phylogenetic analysis generated mostly matching topologies in respect of cumulative datasets from the three corresponding organellar genomes with the associative relationship among the eight families under Cucurbitales order being depicted to be in the sequential array of (Apodanthaceae, Anisophylleaceae, (Cucurbitaceae, ((Coriariaceae, Corynocarpaceae), (Tetramelaceae, (Datiscaceae, Begoniaceae))))). Based on molecular characterization and morphological data analysis, the recircumscribed tribes and genera under Cucurbitaceae were represented, which illustrated a more natural and realistic approach of classifying the family. The novel system comprised a total of ninety five genera placed in fifteen corresponding tribes with five of them being completely new that is Actinostemmateae, Indofevilleeae, Thladiantheae, Momordiceae and Siraitieae.

The genus *Herpetospermum* is placed in Tribe No. 11 named Schizopeponeae. Currently, the genus *Herpetospermum* includes the following three species- *Herpetospermum darjeelingense* (C.B.Clarke) H.Schaef. & S.S.Renner; *Herpetospermum pedunculosum* (Ser.) Baill. and *Herpetospermum tonglense* (C.B.Clarke) H. Schaef. & S.S.Renner.

Herpetospermum pedunculosum is an annual, dioecious, scandent, indigenous, ethno-medicinally significant herbaceous vine of the Tibetan Himalayas employed in the treatment of inflammatory issues in stomach and intestine including haemorrhoids with an ability to withstand the prevailing frigid conditions through increased functioning of antioxidant enzymes in foliage leaves besides significant increment in the ratio of Ascorbic acid to Docosahexaenoic acid in comparison to plants growing in the low altitudinal areas. GC-MS investigation revealed a total of fifty compounds in foliar extracts of *H. pedunculosum* being categorized into varied chemical classes namely carbohydrates, organic acids, lipid derivatives, amino acids, polyamines and secondary metabolites with higher concentration of amino acid constituents detected in samples growing at higher altitudes. Carbohydrates and organic acid components are linked to the reductive pentose phosphate scheme, glycolysis and citric acid cycle; establishing a correlative evidence of adaptability attributes in *H. pedunculosum* with Carbon and amino acid metabologenesis scheme in coping high altitudinal stress concerns. Pure compounds namely Herpetin, Herpetone and Herpetfluorenone extracted from *H. pedunculosum* seeds were evaluated for anti-hepatitis B viral activity through application on HepG2.2.15 cells. Herpetin and Herpetfluorenone exhibited more powerful antiviral activities than Herpetone with Herpetfluorenone being deduced to be less toxic than Herpetin and Herpetone. The complete sequence of *H. pedunculosum* chloroplastid genome is 1,56,531 base pairs (bp) in length, comprising of a pair of inverted repeats (IR's) of size 26,147 bp, one large single-copy (LSC) region of length 85,878 bp in addition to one small single-copy (SSC) region of size 18,359 bp. There are a summative of one hundred twenty eight genes including eighty three protein-

coding genes, in addition to thirty six transfer RNA (tRNA) genes and eight ribosomal RNA (rRNA) genes in the plastid genome of *H. pedunculosum*. On the basis of phylogenetic study with respect to thirteen complete chloroplast genomes of cucurbitaceous species, sisterhood features of *H. pedunculosum* can be concluded with a clade containing *Trichosanthes kirilowii* and *Hodgsonia macrocarpa*; which suggests a close association between tribal rank representatives Schizopeponeae and Sicyoeae in the family Cucurbitaceae.

The previously mentioned information clearly states the quantity of experimental work done with *H. pedunculosum*, along with a significant hint on its medicinal potential. However, no work other than its taxonomic description and geographical distribution has been completed for its sister species *H. darjeelingense* and *H. tonglense*.

DNA barcoding of *Herpetospermum darjeelingense* provided molecular identity to the specimen besides identifying its relatedness to Australian endemic plant *Nothoalsomitra suberosa*, Tibetan sister *Herpetospermum pedunculosum* and *Cucurbita maxima*. Highest similarity of *H. darjeelingense* to an Australian endemic plant can be related to the theory of breaking up of the supercontinent Pangaea with a hint to divergent evolutionary mode including anticipated coexistence of both the plant at the same place at some point of time and gradual splitting apart. The rbcL gene based DNA barcode will now help in accurate identification of the plant. Comparative domain analysis of RBCL peptide sequences of *H. darjeelingense*, *H. pedunculosum* and *H. tonglense* revealed similar domain features with depiction of only low complexity regions initiating from the 8th amino acid and terminating at the 22nd amino acid. Phylogenetic tree construction employing rbcL based gene sequences also disclosed interesting results. The Maximum likelihood type phylogenetic tree infers *H. darjeelingense* to be phylogenetically related to two subclade; first comprising *Cionosicys macranthus*, *Cucurbita ficifolia*, *C. maxima*, *C. moschata* and *C. pepo*; in addition to the second containing *Neoachmandra japonica*, *Corallocarpus boehmii*, *Nothoalsomitra suberosa*, *H. tonglense*, *H. pedunculosum*, *Schizopepon bryoniifolius* and *S. dioicus* whereas, the Neighbor joining based phylogenetic tree depicts *H. darjeelingense* to be closely related to *H. pedunculosum* sequentially followed by *H. tonglense*, *Corallocarpus boehmii*, *Neoachmandra japonica*, *Cionosicys macranthus*, *Schizopepon* spp. (*S. bryoniifolius* and *S. dioicus*), *Nothoalsomitra suberosa* and Cucurbita spp. (*C. ficifolia*, *C. maxima*, *C. moschata* and *C. pepo*).

The plant is habituated to grow at cooler temperatures (<20°C) with relatively high humidity (>50%). Soil condition of samples growing in tea growing areas were observed to be mostly acidic with relatively high moisture content value including elevated levels of organic matter, organic carbon, Nitrogen, Phosphorous, Potassium and Sulphur owing to receiving benefits of proper tea garden management in comparison to soil physicochemical profile of plants surviving in non-tea growing regions, which mostly remain unmanaged and neglected on account of anthropogenic exploitation. The pH of leaf was slightly

alkaline (7.42) including high moisture content. Qualitative analysis suggest the plant to display a fantastic cohesion of bioactive chemical entities ranging from flavonoid, phenolic compounds, cardiac-glycosides, coumarin, steroid, tannin and terpenoids in varying proportions.

Quantitative assay depicts remarkable antioxidant potential of *Herpetospermum darjeelingense* solvent extracts. The experimental findings clearly indicate that the percentage of DPPH induced free radical neutralizing capability of sample concentrates exhibited an increment towards the polar end owing to greater solubilization of anti-oxidant molecules in the polar solvents. Acetone extract showed higher Ferric reducing ability in comparison to Methanol based sample concentrates whereas highest flavonoid content was recorded in ethanol extracts with lowest being portrayed in methanolic sample concentrates respectively. Promising Nitric oxide and Hydrogen peroxide quenching potential including high Phenolic content was noted in sample extracts. The antimicrobial efficacy of *H. darjeelingense* methanolic extract was very mild with no noteworthy observation. However, the anti-microbial effectiveness of Silver nano formulations exhibited significant elevation in comparison to normal sample based methanolic extracts. Observance of increase in DPPH induced free radical quenching activity of Silver nano formulations in comparison to normal plant extract further affirms the immense potential of biogenic Silver nanoparticles of *H. darjeelingense* as anti-oxidant agents in addition to exhibition of significant anti-lipid peroxidation potential. GC-MS analysis deciphered a total of sixty bioactive compounds across multiple collection spots depicting biochemical variability. The percentage of anti-oxidant and anti-cancer compounds contained in the endemic cucurbit is noteworthy with more than 95% bioactive metabolites. The signature compounds detected in the methanolic extract include the likes of 03027205002 Flavone 4'-OH,5-OH,7-di-o-glucoside, Quinic acid, Phytol, Methyl linolenate, Gamma-sitosterol (Clionasterol), Methyl palmitate, Phytyl palmitate, Squalene and β-Sitosterol. The maximum amount of underlying biochemical compounds as explored through GC-MS analysis belonged to the group of terpenoids followed by fatty acid derivatives and flavonoid. Therefore, GC-MS investigation of *Herpetospermum darjeelingense* uncovered the existence of various bioactive compounds possessing anti-inflammatory, anti-diabetic, hepatoprotective, anti-Alzheimer and anti-microbial properties. The variability range of compounds across tea garden regions were more than non-tea garden areas; however more bioactive compounds were noted in non-tea garden areas. The non-tea garden regions exhibited more bioactive compounds owing to higher levels of ecological stress leading to synthesis of biologically relevant molecules for countering the stress issues. Plants of the same locality possessed higher correlation values besides clustering together in NTSYS generated dendrogram in respect of constituent biochemical entities than other locale proving that they either belonged to a similar genetic stock or ecological influence has modulated synthesis of these metabolites. Now, it was a question whether those bioactive compounds were metabolized in the plant

system either due to chance factors, or biotic and abiotic stress mechanisms play a role in their biosynthesis or do they have a specific biological pathway? The answers revealed seems interesting as all the bioactive compounds seem to follow a specific and ordered metabologenesis pathway that may be essentially required for the survival of the plant. The metabolomic pattern revealed three major pathway schemes operating in the plant with respect to the bioactive compounds namely the fatty acid biogenesis model, terpenoid biosynthesis scheme (Mevalonic acid and Methyl erythritol phosphate pathway) and the Shikimic acid pathway for generation of the major biomolecular entities. In the whole, the plant utilizes maximum of its available energy towards biosynthesis of these bioactive metabolites leading to metabolomic thermodynamic exhaustion under a strict level of genetic control. The plants possessed antidiabetic, hepatoprotective compounds as well; which exhibited excellent results through *in silico* and *in vitro* analysis. Four pure compounds were isolated namely Beta-sitosterol, Lupeol, Betulinic acid and Cerin; among which Cerin exhibited excellent *in silico* results against proteins associated with breast and lung cancer, while Beta-Sitosterol showed better results in *in vitro* assay against breast (MDA-MB-231) and lung (HOP-62) cancer related cell lines. Physicochemical characterization of isolated pure molecules depicts β-Sitosterol to possess the highest drug-likeness model score among the test molecules in addition to ADMET prediction indicating Cerin to be the best drug molecule. The conduction of tissue culture methodology in respect of *ex situ* conservation will help in rapid multiplication of the species, thereby helping in preservation of germplasm. The plants responded well under tissue culture conditions; however cold weather is a requisite for its survival post hardening of plantlet; demonstrating prospects of application of *in vitro* technique for regeneration and augmentation of the cucurbit climber in its natural habitat. Metabolic comparison between naturally growing and *in vitro* regenerated plant portrayed fascinating results with recognition of primary metabolite Guanosine in addition to triterpenoid sterol Cholesterol and tetraterpenoid biomolecule Retinol in the tissue cultured plantlet as a replacement of cardinal bioactive metabolic entities investigated in the naturally growing plants.

The foremost restraint in tissue culture was the assault of an endophytic fungus. The concealed endophyte was identified and confirmed to be *Fusarium verticillioides* through molecular techniques. GC-MS analysis revealed a decline of essential biomolecules with few compounds conferring antifungal property to be completely switched off including upregulation of some specific antifungal elicitors in endophyte containing plant. A number of cardinal antifungal stress compounds detected in endophyte containing sample extracts were synthesized by compensating varied bioactive (Terpenoid and long odd chain fatty acid derivatives) molecular entities towards equilibration of metabolic expenditure. The metabolic crosstalk between endophyte *Fusarium* and host *Herpetospermum* was investigated to exert enormous biotic stress unsettling the total biomolecular assembly of the host plant; as a result challenging the overall wellbeing

of the plant. Therefore, it could be hypothesized that the hidden endophyte, *Fusarium verticillioides* plays a key role behind diminishing of *H. darjeelingense* population in Tea gardens of Darjeeling hills, through antagonization of bioactive molecules essential for its continued existence besides anthropogenic factors. Additionally, *Fusarium* is a soil borne fungus prevailing in tea garden soil which further indicates that *H. darjeelingense* plants located in tea garden regions will be more prone to fungal invasion than non-tea garden areas.

The research would bridge the gap between traditional ethnomedicinal knowledge and concealed bioactive principles in medicinal plants. The molecular data retrieved from this plant can help to solve phylogenetic riddles of the Gourd family. Whole genome analysis can aid in understanding the overall metabolomic blueprint of the plant with a clue towards likely models of bioengineering technology towards directional production of a valued therapeutic metabolite. The investigated biogenesis pathway could help to screen and isolate a range of biologically significant molecular constituents in a voluminous scale depending on other complimentary aspects providing a vivid idea of how metabologenesis of bioactive metabolites occur through a stringent mechanism of genetic control. Just four pure molecules possessing anticancer activity could be isolated with prospective potential of obtaining a greater numeral of anti-proliferative compounds from *Herpetospermum darjeelingense*. Isolation, screening and sequential pharmaceutical trial employing *in silico*, *in vitro* and *in vivo* methodology can revolutionize the global futuristic approach to cancer research. Implementation of conservation strategies through tissue culture technique can be extensively practiced to preserve these ethnomedicinally imperative floral treasures anticipated to alleviate other complex disorders currently way afar the thoughts of current medicinal research. The research can also help in disentanglement of the conception behind reasons of species endangeredness through investigation of endophytic traits.