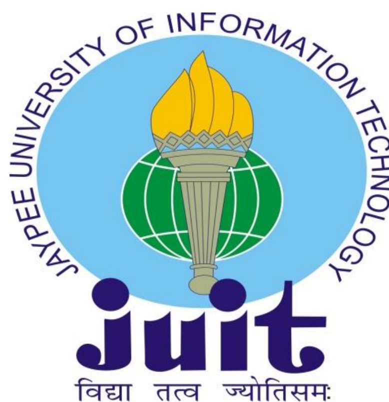
GENOME AND METABOLOMIC BASED APPROACHES TO AUTHENTICATE *Swertia chirata* FROM ITS ADULTERANTS AND THEIR COMPARATIVE EVALUATION AGAINST HYPOXIA INDUCED OXIDATIVE STRESS IN RATS

Thesis submitted in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

KRITIKA KAUSHAL



DEPARTMENT OF BIOTECHNOLOGY & BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, DISTRICT SOLAN, H.P., INDIA

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D thesis entitled **"Genome and Metabolomic based approaches to authenticate *Swertia chirata* from its adulterants and their comparative evaluation against hypoxia induced oxidative stress in rats"** submitted by **Ms. Kritika Kaushal** at **Jaypee University of Information Technology, Waknaghat, India,** is the record of candidate's own work carried out by him under our supervision. This work has not been submitted elsewhere for any other degree or diploma.

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled **"Genome and Metabolomic based approaches to authenticate *Swertia chirata* from its adulterants and their comparative evaluation against hypoxia induced oxidative stress in rats"** submitted at **Jaypee University of Information Technology, Waknaghat, India** is an authentic record of my work carried out under the supervision of **Dr. Anil Kant**. I have not submitted this work elsewhere for any other degree or diploma.

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Dedicated to my Parents

This thesis is dedicated to my caring and devoted parents, for all their love, support and encouragement. They have been a source of inspiration for me to do my best and helped me sail through difficult chapters of my life. They have been ultra-supportive in all decisions in my life and this thesis is dedicated to their efforts and sacrifices.

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(Kritika Kaushal)

TABLE OF CONTENTS

	Page No.
List of Tables	vi
List of Figures	vii
List of Abbreviations	ix
Abstract	**1-3**
Chapter 1 : Introduction	**4-10**
1.1. Distribution	
1.2. Taxonomy of *Swertia chirata*	
1.3. Period of occurrence	
1.4. Habitat and cultivation	
1.5. Morphological features	
1.6. Phytochemistry	
1.7. Pharmacological importance	
1.8. Adulteration and substitution	
1.9. DNA barcoding	
1.10. Metabolomics based profiling of *Swertia chirata* and its adulterants	
1.11. Medicinal plants and oxidative stress	
Chapter 2 : Review of Literature	**11-27**
2.1. S*wertia chirata*	
2.2. Overview of the botanical description of *S. chirata*	
2.3. Trade and conservation status	
2.3. Trade and conservation status	
2.4. Phytochemical profile of *S. chirata*	
2.6. Medicinal properties of *S. chirata*	
2.6.1. Ethnomedicinal value	
2.6.2. Recent research on the medicinal values of *S. chirata*	
2.6.2.1. Anti-diabetic properties	
2.6.2.2.Anticancer properties	

2.6.2.3. Anti-malarial properties
2.6.2.4. Hepatoprotective properties
2.6.2.5. Anti-inflammatory properties
2.6.2.6. Antimicrobial properties
2.6.2.7. Antioxidant properties
2.6.2.8. Anti-leishmanial activity
2.6.2.9. Analgesic and antipyretic properties
2.6.2.10. Gastroprotective properties
2.6.2.11. Central nervous system (CNS) modulatory activity
2.6.2.12. Potential as a hair tonic
2.7. Oxidative stress

Chapter 3 : Investigation of the use of candidate DNA barcodes in discriminating *Swertia chirata* from its adulterants **28-45**
3.1 Introduction
3.2 Material & methods
3.2.1 Plant material
3.2.2 DNA extraction, amplification, and sequencing
3.2.3 Sequence alignment and data analysis
3.3 Results
3.3.1. Amplification and sequencing of purified amplicons
3.3.2. Nucleotide polymorphism and Primer Performance
3.3.3. *In silico* analysis to calculate Inter specific and Intra specific divergence
3.3.4. Estimation of evolutionary divergence
3.4. Discussion
3.5. Conclusion

Chapter 4 : Comparative Metabolomics Reveals the Metabolic Variation between *Swertia chirata* and its Adulterants **46-59**
4.1 Introduction
4.2 Materials and Methods
4.2.1 Plant Materials and its Identification

4.2.2 Preparation of Extracts
4.2.3 Metabolite Profile of *S. chirata* and ITS ADULTERANTS
4.2.4 Statistical Analysis
4.3 RESULTS
4.3.1 GC-MS-Profiling
4.3.2 Statistical Analysis of GC-MS based Metabolomic Data
4.3.2.3. Phylogenetic Analysis
4.3.2.4 . Principle Component Analysis (PCA) Analysis
4.3.2.5 . Variable Importance Projection (VIP) Analysis
4.4 Discussion
4.5 Conclusion

Chapter 5 : Antioxidant and hepatoprotective effect of *Swertia chirata* on hypoxia-induced oxidative stress in wistar rats **60-73**
5.1 Introduction
5.2 Materials and methods
5.2.1 Material
5.2.2 Sample collection and extraction
5.2.3 Animals
5.2.4 Animal groupings
5.2.5 Induction of hypoxia
5.2.6 Animal sacrifice and sample collection
5.2.7 Biochemical evaluation
5.2.7.1 Mitochondria isolation
5.2.7.2 Mitochondrial ROS generation
5.2.7.3 Lipid peroxidation
5.2.7.4 Catalase activity
5.2.7.5 Glutathione peroxidase assay
5.2.8 Liver function test
5.2.9 Histopathological examination
5.2.10 Statistical analysis
5.3 Results

5.3.1 Extractive yield and phytochemical evaluation
5.3.2 Biochemical investigation
5.3.2.1 Mitochondrial ROS generation
5.3.2.2 Lipid peroxidation
5.3.2.3 Catalase activity
5.3.2.4 Glutathione peroxidase assay
5.3.3 Liver function tests
5.3.4 Histopathological examination
5.4 Discussion
5.5 Conclusion

Chapter 6 : Hydroalcoholic extract of *Swertia chirata* and *Swertia cordata* attenuate hypoxia mediated memory dysfunction by improving neuronal survival in Wistar rats **74-91**
6.1. Introduction
6.2. Material and Methods
6.2.1. Chemicals and Reagents
6.2.2. Plant Collection and Extract Preparation
6.2.3. Animals
6.2.4. Animal Groupings
6.2.5. Hypoxia Induction
6.2.6. Rota rod Test
6.2.7. Memory Function Tests
6.2.7.1. Passive Avoidance Test
6.2.7.2. Active Avoidance Test
6.2.8. Animal Sacrifice and Sample Collection
6.2.9. Mitochondrial Reactive Oxygen Species (ROS) Generation
6.2.10. Levels of Lipid Peroxidation
6.2.11. Catalase Activity
6.2.12. Glutathione Levels
6.2.13. Histopathological Examination
6.2.14. Statistical Analysis
6.3. Results

6.3.1. Extractive yield and Phytochemical Evaluation
6.3.2. Behavioral Investigation
6.3.2.1. Rota Rod Test
6.3.2.2 . Passive avoidance Test
6.3.2.3 . Active avoidance Test
6.3.3. Biochemical Investigation
6.3.3.1.Mitochondrial Reactive Oxygen Species (ROS) Generation
6.3.3.2.Lipid Peroxidation
6.3.3.3.Catalase Activity
6.3.3.4.Glutathione Level
6.3.4. Histopathological Examination
6.4. Discussion
6.5. Conclusion
Overall Conclusion and Future Directives **93**
References **95-114**

LIST OF TABLES

Table No.	Caption	Page No.
2.1	Common names of *S. chirata* in different languages	14
2.2	Herbal formulations which has *S. chirata* as a main therapeutic ingredient	16
2.3	List of secondary metabolites present in *S. chirata* (adapted from (Kumar & Van, 2015; Joshi and Dhawan 2007)[1, 29]	21
2.4	Ethnomedicinal use of *S. chirata* (adapted from Joshi and Dhawan 2007) [29].	23
3.1	Sampling localities and number of samples collected from each location	32
3.2	Primer sequences, optimized annealing temperature and size of amplicon obtained w.r.t. candidate regions	35
3.3	Accession Numbers of Sequences of candidate regions of species submitted in NCBI, Nucleotide database	36
3.4	Nucleotide polymorphism obtained in PsbK-I target region	38
3.5	Output of Primer Plex to target the nucleotide polymorphism of psbK-I region	39
3.6	Estimation of evolutionary divergence in sequences of psbK-I region.	42
4.1	Potential Marker compounds which can be used to discriminate different pair of the *Swertia* species and adulterants	52
4.2	Most Potential marker compounds which can be used to differentiate *S. chirata* from adulterant species	52

LIST OF FIGURES

Figure No.	Legend	Page No.
1.1	*Swertia chirata* (a) Plant in natural habitat; (b) flower; (c) dried form of plant; (d) Powdered form in herbal drug industry.	6
1.2	Plants of (A) *Swertia angustifolia*; (B) *Swertia ciliata*; (C) *Swertia cordata*; (D) *Swertia chirata*	8
2.1	Geographical distribution of *S. chirata*: Shaded region represent the Himalayan regions which are the natural habitat of *S. chirata*[1]	13
3.1	Map of Himachal Pradesh and Uttarakhand from where S. *chirata* and other plant were collected	32
3.2	PCR amplification Product obtained in case of target region A) rpoB; B) rpo C	37
3.3	PCR amplification Product obtained in case of target region A) rbcl; B) Mat K	37
3.4	PCR amplification Product obtained in case of target region A) psbK-psbI; B) psbA-trnH	37
3.5	Analyses of the inter-specific divergence between congeneric species and intra-specific variation of the six loci.	40
3.6	K2P distance NJ tree for psbK-I . A consensus NJ tree for *swertia chirata* and its adulterants assessed with 1000 bootstrap replicates was constructed by bootstrap analyses with the bootstrap value indicated at the branches (bootstrap values of less than 50 are not shown).	43
4.1	Standard GC-MS Chromatogram showing different peaks at different retention time	51
4.2	Number of common and uncommon metabolites in different species with respect to *Swertia chirata*	51
4.3	Dendrogram showing the evolutionary relationship between different species: *Swertia ciliata, Swertia chirata, Swertia chordata, Andrographis paniculata* and Mixture of all species.	54
4.4	Comparative PCA analysis of the metabolites of four species i.e *S. Cordata,* 2- *S. Chirata,* 3- *A. Paniculata,* 4- *S. Ciliata* as Scatter plots	55

4.5	Principle component analysis (PCA) score plot (a) and variable importance in projections (VIP) scores (b) of 3 species of *Swertia*. The VIP plot is sorted in descending order. Colored boxes: concentrations of the corresponding metabolite. Dash line: critical value.	56
5.1	Effect of *S. chirata* treatment on hypoxia induced oxidative stress	69
5.2	Effect of *S. chirata* treatment on hypoxia induced hepatic damage (Liver Function Test).	70
5.3	Effect of *S. chirata* treatment on hypoxia induced liver damage. Histopathological examination of liver using hematoxylin-eosin staining.	72
6.1	Effect of *S. chirata* and *S. cordata* treatment on hypoxia mediated behavioral alterations	84
6.2	Effect of *S. chirata* and *S. cordata* treatment on hypoxia induced oxidative stress	87
6.3	Effect of *S. chirata* and *S. cordata* treatment on hypoxia induced neuronal damage.	90

LIST OF ABBREVIATIONS

CBOL	Consortium for the Barcode of Life
mat K	Megakaryocyte-Associated Tyrosine Kinase
rcbL	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
rpoB	beta subunit of RNA polymerase
rpoC1	DNA-dependent RNA polymerase
CNS	central nervous system
NTFPs	non-timber forest products
TLC	Thin Layer Chromatography
NGS	Next Generation Sequencing
GIT	gastro intestinal tract
CCRAS	Central Council for Research in Ayurvedic Sciences
GSH	glutathione
ROS/RNS	reactive oxygen and nitrogen species
PCA	Principle component analysis
VIP	importance in projections
DTNB	dithiobisnitrobenzoic acid
TBA	thiobarbituric acid
BSA	bovine serum albumin
HEPES	mM hydroxyethyl piperazine ethane-sulfonic acid
HYP	Hypoxia
ANOVA	Analysis of Variance
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
HPLC	High performance liquid chromatography
DTNB	5,5'-Dithiobis-(2-Nitrobenzoic Acid)
SGOT	Serum glutamic-oxaloacetic transaminase
ALP	serum aspartate transaminase
SGPT	serum glutamic pyruvic transaminase
CTRL	Control
MDA	Malondialdehyde
SPSS	Statistical Package for Social Sciences
UPGMA	Unweighted Pair Group Method with Averages

ABSTRACT

ABSTRACT

The plant based drugs plays a vital role in healthcare system in developing as well as developed countries and herbal medicines are considered comparably safe. The adulteration and substitution of herbal drugs is the major problem causing threat to the herbal drug industry and to the research on commercial natural products. *Swertia chirata* of family Gentianaceae is widely used in many traditional systems of medicines because of its immense pharmacological properties. However, high market value and similar morphological characteristics of *Swertia* species, many adulterants of *S. chirata* are available in the market which goes by the name of chirata.

There are no standard method or global standard protocols are available presently to check adulteration and to ascertain plant species or plant parts used in herbal medicines in general and that of *S. chirata* in particular. So in current investigation we have made an attempt to develop a method of *S. chirata* identification based on DNA bar coding a recently used tool for species identification as well as chemical profiling. Since no comparative study of chemical profiles and medicinal activities of the adulterant species *S. chirata* has been performed so for. So In this investigation we have conducted a comparative metabolomics based profiling of *S. chirata* and its adulterants

Differential identification of four adulterant species along with nine *Swertia* species were examined by using six DNA candidate barcodes (rbcL, matK, atpF-H, rpoB, ITS, psbK-I). The results suggest that inter specific divergence was high than intra specific divergence in case of psbK-I when compared to rest of the regions. Also, the single nucleotide polymorphism obtained in psbK-I region and the Neighbor-joining tree obtained by Kimura 2-parameter model, method further supported the potential of the region to be used as barcode to identify *S. chirata* and differentiate it from most commonly used adulterants. So it is concluded that barcode psbk-I can be used proficiently to identify *S. chirata* and its adulterants.

The adulterant species of *S. chirata* were analyzed to identify the metabolites that can differentiate and discriminate the same from its adulterants. The results indicate that Tetradecanal, Methyl 3-hydroxy-2-oxobutanoate, 1, 2-benzenedicarboxylic acid are the most

potential marker compounds to differentiate *S. chirata* from its adulterants. However a separate detailed study is required to standardize workable protocol. Phylogenetic analysis based on GC-MS generated metablomic data revealed that *S. cordata* is more closely related to *S. chirata* in comparison to *Andrographis paniculata.* In PCA analysis samples with same metabolomic compositions were clustered together and are represented as a separate cluster, while those with different metabolic components were shown as dispersed. By utilizing these approaches, it is possible to efficiently identify not only the presence of adulterants in the herbal preparations of S. *chirata,* but these techniques will also allow us to identify the degree of adulterations.

S. chirata is used in traditional medicine to treat several diseases most importantly related to liver, neurotoxicity, diabetes, Alzheimer's disorder, etc. However, mechanism of hepato-protective and other activities is scare. Oxidative stress is root cause of occurrence and progression of a myriad of complications. So aim of one aspect of present study was to assess the hepato-protective and antioxidant potential *S. chirata* in hypoxia induced hepato-compromised rats. Liver histopathology confirmed the marked hepatic damage induced by hypoxia and revealed that *S. chirata* efficiently rescued liver from hypoxic damage. Hydroalcoholic extract of *S. chirata* is a potent hepatoprotective intervention which was associated with its ability to attenuate oxidative stress and improve liver functions.

The neuromodulatory potential of *S. chirata* and *S. cordata* during hypoxia-induced neuronal damage in Wistar rats was also investigated and to determine the underlying mechanism. Hypoxia treatment resulted in marked increase in oxidative stress as indicated by the significantly elevated reactive oxygen species and lipid peroxide levels. *S. chirata* and *S. cordata* pretreatment prevented hypoxia induced oxidative stress generation in the hippocampus region of the rat brain. Pretreatment with plant extracts efficiently rescued rat neuronal cells from hypoxic damage. In comparison between the two plant extracts, *S. chirata* extract pretreatment was observed to have better neuromodulatory effect than *S. cordata.*

CHAPTER 1
INTRODUCTION

1. INTRODUCTION

There has been tremendous surge in acceptance and reliance on herbal medicine based healthcare system in last couple of years. According to a recent estimate of World Health Organization 80% of population depend on herbal medicines one or another aspect of primary health care needs. The contribution of plant based drugs in developed countries is around 25%, whereas as in developing contraries like china and India, it has been estimated to be up 80% of total drugs used, as per world health organization. The rural population of developing countries largely depends on indigenous systems of medicine for their primary health care. Herbal medicines are considered comparably safe with minimal side effects in most of world's traditional system of medicines.

Among plants often used in traditional medicine, *Swertia chirata of* Gentianaceae family plays a vital role. It is an ancient herb which was introduced to Europe in 1839. Roxburgh, first described *Swertia*, a genus in family Gentianaceae as *Gentiana chyra*yta in 1814 [1, 2]. There are about 135 species in this genus which are annual or perennial herbs. *Swertia chirata* is considered to be most important amongst 40 species of *Swertia* found in India due to its pharmacological properties [3, 4]. *S. chirata* has been reported to be useful in treating liver disorders, malaria, and diabetes [5]. Numerous herbal remedies contain various *Swertia* species as common ingredient. The plant has reached at the verge of extinction due to its high demand and overharvesting from wild populations usage of mostly underground tissues, which require uprooting, combined with habitat destruction.

1.1 DISTRIBUTION

S. chirata is distributed from Kashmir to Bhutan at an altitudinal range of 1,200-3,000 m in temperate Himalayas and in Khasi hills of Meghalaya at 1200- 1500 m [1, 6]. Plant is endangered and is on the verge of extinction as result of over-exploitation from its natural habitats due to its widespread uses in traditional medicines.

1.2 HABITAT AND CULTIVATION

The plant grows well in semi-shade, swampy conditions and requires damp soil. The woodland forests with sunny edges and partial shade as well as marshy lands are favorable for *chirata* to bloom and flourish. The areas where summers are cool are best suited to the plants as these can withstand temperatures as low as -15°C during winters. The plant rapidly spreads from seeds which are shed during October and November. Seeds are very small in size, should be sown in nursery and seedlings transplanted later to the field [7]. The herb can

be cultivated in suitable localities in the temperate Himalayas. It is propagated by sowing seed in spring season, when the temperature is below 10° C. Seedlings grown enough to be handled, are transplanted in the field or separate pots. Plants normally grow up to a height of one meter and plant bloom in the months of September to October. Harvesting is when seeds begin to set in and uprooted plants sun dried [8].

1.3 MORPHOLOGICAL FEATURES

Leaves of *S. chirata* are broad, around 10 cm long, lanceolate, opposite, sessile, acute at tip which have five nerves (Fig 1.1a). Large leafy panicle inflorescence is observed which attain a height of approximately 143 cm. and are greenish yellow with tinge of purple. Fruits an ovoid yellowish brown unilocular many seeded capsule, and are very bitter in taste [9]. Roots are about 5-10 cm long and light yellowish to brown in colour.

The stem is yellowish or purplish brown in colour with thin readily separable bark. It is cylindrical in basal region, quadrangular with ascending branches, wood of stem is porous and yellow, enclosing in the intermodal region.

Figure 1.1: *Swertia chirata* (a) Plant in natural habitat; (b) flower; (c) dried form of plant; (d) Powdered form in herbal drug industry.

1.4 PHYTOCHEMISTRY

S. chirata contains chiratin and ophelic acid which are amorphous or crystalline substances. It also contains Xanthones swerchirin, glycoside amarogentin swertiamarin, flavonoid mangiferine, etc. Taxonomically informative molecules such as irrioids, xanthones, mangiferin and C-glucoflavones are responsible for its broad spectrum medicinal properties [8-11].

1.5 PHARMACOLOGICAL IMPORTANCE

Due to the presence of its active chemical constituents *S. chirata* holds a valuable position in Indian medicinal system. It is used in household remedies as expectorant digestive, blood purifier, antidiarrhoetic, febrifuge, anthelmintic, antiperiodic and carminative etc. In ayurveda system of medicines *S. chirata* is reported to be used as anthelminitic antipyretic, antiperiodic, and in ailments like asthma and leucorrhoea [4]. Chirata extract is important constituents of many herbal medicines e.g. Ayush-64, Diabecon, Menstrual syrup and Melicon-V ointment [7, 12] and some veterinary medicines and is reported to impart flavor to cattle feed [13]. The roots are considered to be the most important part for its medicinal properties [4] though entire plant has medicinal properties.

1.6 ADULTERATION AND SUBSTITUTION

Medicinal herbs have been used in one or another form by indigenous system of medicine like Ayurveda, Sidha and Unani. Indeed approximately 70% of synthetic medicines are derived from plants. Rapid increase of approximately 15% annually has been seen in the global economy of herbal products and the alternative medicine market [14]. New herbal products are being launched in herbal drug industry. However, one of threat to the herbal drug industry, its growth and adaptation is adulteration and substitution of herbal drugs. The plant substitutes with inferior of no therapeutic potential as that of original drug are used as adulterant. Due to rampant malpractice of adulteration for profits, increased concern are being raised about safety and efficacy of herbal products, which were once considered as safe and free from side effects [15,16]. Due to scarcity and high demand in pharmaceutical industry of *S. chirata* the other *Swertia* species such as *S. angustifolia, S. paniculata, S. ciliata, S. cordata* and unrelated species *Andrographis paniculata* are often being mixed with *S. chirata*, misused or being used as substitutes of *S. chirata*. This is affecting the trade and economics of *S. chirata*. Adverse consequences of species adulteration on the health and safety of consumers have also been reported recently [12].

Fig 1.2: Plants of (A) *Swertia angustifolia*; (B) *Swertia ciliata*; (C) *Swertia cordata*; (D) *Swertia chirata*

There are no standard method or global standard protocols are available presently to check adulteration and to ascertain plant species or plant parts used in herbal medicines in general and that of *S. chirata* in particular. For authentication of *S. chirata* a few criteria and traditional methods have been developed which are mainly based on phenotypes and analysis of chemical components. The adulterants of *S. chirata* closely resemble with it in plant and flower morphology (Fig 1.2) and have many common chemical compound in their chemical profile. Moreover the methods based on morphological identification may not be applicable in case of powdered herbal preparations (Fig 1.1 C, D). Similarly the method based on chemical components are also difficult to apply given that fact that may of adulterants may also contain some of key chemical constituents unless chemical profiling is comprehensive enough.

Considering the facts presented above morphological identification and method based on chemical analysis available so for are not enough for accurate identification of *S. chirata*, and quality of *S. chirata* supply in the markets and *S. chirata* preparations. So in current investigation we have made an attempt to develop a method of *S. chirata* identification based on DNA bar coding a recently used tool for species identification.

1.7 DNA BARCODING

In recent years, the new diagnostic technique known as DNA barcoding has evolved which can be used as an efficient tool for the species identification [17]. This approach includes sequencing of short standard DNA region from a small fragment of genome, which can be amplified by universal primers [18]. Consortium for the Barcode of Life (CBOL), Plant Working Group has recommended several regions that can be used as candidate barcodes for plants. Some are non-coding spacers of coding gene Megakaryocyte-Associated Tyrosine Kinase (mat K), ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rcbL), beta subunit of RNA polymerase (rpoB) and DNA-dependent RNA polymerase (rpoC1) [18,19]. Whereas, some are on some multi-region combinations such as

combinations of rbcL and matK, trnH-psbA+ rbcL. In this investigation, six candidate barcodes (ITS, psbA-trnH, matK, and rbcL) from plastid and nuclear genome were evaluated to authenticate *S. chirata* and its adulterants. The primary focus of the study was to develop a species identification method for *S. chirata* which can discriminate *S. chirata* from its adulterant species. Therefore, keeping in view the current medicinal status of *S. chirata,* the present study is an attempt to evaluate some of the highly recommended plant DNA barcoding loci to determine their efficiency to be used for the identification and authentication of *S. chirata* from its adulterants.

As mentioned earlier also *S. chirata* species has reached to the verge of extinction due over exploitation of species from the wild resources. This is one of the reason of using related *Swertia sp.* and other non *Swer*tia species with similar chemical properties as adulterants, which raises two questions.

1. Is it possible to discriminate *S. chirata* from its adulterants on the basis of chemical profiles or key chemical constituents?
2. If chemical profile of adulterants is somewhat similar, is it possible use these plant species as really substitutes of *S. chirata* which near to extinction?

Since no comparative study of chemical profiles and medicinal activities of the adulterant species *S. chirata* has been performed so for. So In this investigation we have conducted a comparative metabolomics based profiling of *S. chirata* and its adulterants

1.8 METABOLOMICS BASED PROFILING OF *SWERTIA CHIRATA* AND ITS ADULTERANTS

Plants produce variety of secondary metabolites, such as glycosides, alkaloids, tannins, saponins etc., which possess variety of pharmacological activities and have been used for the treatment of various ailments since ages. Medicinal plants belonging to same family or some genus shown remarkable resemblance in their physical properties, and to some extant in their medicinal values. Recently, several techniques and technologies have been developed for the analysis and differentiation of plant material based on their phytochemical constituents, and one such extremely useful technique is omic-technique. In recent times, there has been remarkable advancement in the techniques like Gas-chromatography, Liquid chromatography, Mass spectroscopy etc., which has made it feasible to separate the individual components of herbal material, identify it, analyze it and differentiate various herbal preparations in the basis of received outcome. In the present study

the adulterant species of *S. chirata* were analyzed to identify the metabolites that can differentiate and discriminate the same from its adulterants.

1.9 MEDICINAL PLANTS AND OXIDATIVE STRESS

Oxidative stress is root cause of occurrence and progression of a myriad of complications such as hepatotoxicity, neurotoxicity, diabetes, Alzheimer's disorder, etc. [20]. Also, despite well-known association of the hypoxia and oxidative stress with the development of neurological complications, there is not even single drug available till date for the management of oxidative stress associated complications. Therefore, some alternative and safer treatment strategy is urgently needed which can alleviate oxidative stress during hypoxia and other conditions like diabetes, chronic stress etc. and rescue neurons from degenerations and associated complications.

S *chirata* is used in traditional medicine to treat several diseases most importantly related to liver [21]. However the literature on experimental validation and mechanism of hepato-protective activity is scare. So aim of one aspect of present study was to assess the hepato-protective and antioxidant potential *S. chirata* in hypoxia induced hepato-compromised rats.

The used of *S. chirata* and related species in e mental disorders relatively unexplored so far, besides, the mechanisms through which the beneficial effects on brain are achieved also unknown. Therefore, the last leg of the present study was designed to get and insight to the neuromodulatory effect of *S. chirata* and *S. cordata* during hypoxia in Wistar rats. At the same time the comparative analysis of effects of extracts of *S. chirata* and *S. cordata* on hypoxia mediated memory dysfunction in rats also answer the question posed, whether adulterant *S. cordata* can really be used as substitutes of *S. chirata*.

Thus, considering the above mention problems the following objectives were designed as guiding principles of present study:

1. Investigation of the use of candidate DNA barcodes in discriminating *S. chirata* from its adulterants.
2. Comparative phytochemical profiling to evaluate the metabolic variation among the *S. chirata* and its adulterant species.
3. Antioxidant and hepato-protective effect of *S. chirata* on hypoxia-induced oxidative stress in male Wistar rats.
4. To investigate the effect of hydroalcoholic extract of *S. chirata* and *S. cordata* on hypoxia mediated memory dysfunction in Wistar rats.

CHAPTER-2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Natural products have been the integral part of the health care system of every civilization throughout the world, for both humans and animals. The use of medicinal plants has gained much more importance in recent time due to the increasing health care cost, severe side effects of allopathic medicines and resistance to the available drugs [22]. A report of WHO estimates that 80% population residing in the developing countries relies on natural products to meet their health care needs. Moreover, approximately 25% of modern drugs standards such as pharmacopoeias are derived from plants and there are several drugs in them which are the synthetic modifications or analogues of plant derived molecules [23, 24]. Every plant produces its characteristic metabolites which may be same in different species or may vary depending upon the environmental conditions in which the plant grows. This wide distribution of plant metabolites result is widespread and different medicinal values of each plant and they are thus used in the management of several ailments [25]. Primary or secondary metabolites present in plants are derived from many precursor molecules by various primary metabolic processes. Secondary metabolites are responsible for the bioactivity of a plant and they can be categorized primarily into terpenoids, alkaloids, glycosides, phenolic compounds, fatty acids, saponins, tannins, etc. The medicinal value of a plant lies in its secondary metabolites and these metabolites are needed for the formulation of herbal drugs. This not only increases the demand of these plants but attracts adulterants and substandard products also, which are very difficult to deal with [26].

Owing to the tremendous medicinal importance of *S. chirata*, both in modern medicines and traditional system of medicines throughout the world, in this section we have provided a detailed description of this herbal plant, besides, discussing its medicinal and economic significance.

2.1 *SWERTIA CHIRATA*

Genus *Swertia* belongs to Gentianaceae family of plants. This family includes large number of annual and perennial herbs which represents about 135 species in total. Plants belonging to *Swertia* species have been used extensively number of herbal formulations available today. So far, a total of 40 species of *Swertia* has been reported in India [4, 27]. Amongst these species, *S. chirata* (Roxb. ex Fleming) is the most important herb pertaining to medicinal value, because of which this plant is widely used in herbal preparations. *S.*

chirata was firstly described in 1814 by Roxburgh and at that time it was named *Gentianachyrayta* [2]. Due to its high medicinal value, this plant has been subjected to overexploitation in the wild, because of which it is now categorized as a critically endangered medicinal herb and is on verge of extinction. It is found at an altitude of 1200-2200 m in high altitudes of Himalayas, ranging from mountains of Kashmir to the valleys of Bhutan (Figure 2.1) [27, 28].

Figure 2.1: Geographical distribution of *S. chirata*: Shaded region represent the Himalayan regions which are the natural habitat of *S. chirata* [1]

It is known by different names in different languages (Table 2.1). In Sanskrit *S. chirata* is known as Anaryatikta, Bhunimba, Chiratitka and Kairata. In Arabic and Farsi languages this plant is commonly known as Qasabuzzarirah. *S. chirata* is known as Sekhagi in Burma, Chiaravata in Urdu, and chirata in Nepali language [29]. The plant can grow as annual or perennial, probably depending upon the environmental conditions in which it grows. Therefore, some literature reports this plants as an annual herb [4, 30], while different group of scientists have reported it as a biennial or perennial herb [31].

Table 2.1: Common names of *S. chirata* in different languages

S. No.	Language	Common Names
1.	Assamese	Chirta
2.	Arabic and Farsi	Qasabuzzarirah
3.	Bengali	Chirata
4.	Burmese	Sekhagi
5.	English	Chireta
6.	Gujrati	Kariyatu, Kariyatun
7.	Hindi	Chirata, Charaita, Chirayata
8.	Kannada	Chirata Kaddi, Chirayat, Nalebevu
9.	Kashmiri	Chiraita, Lose Lepcha Rung kyen
10.	Punjabi	Chiraita, Chiretta
11.	Malayalam	Kirayathu, Nilamakanjiram, Nelaveppu, Uttarakiriyattu
12	Marathi	Charayatah, Chirayita, Kaduchiraita, Kiraita
13.	Nepalese	Chiraita, Chiraito, Chirrato, Kairata, Lektite, Timda, Tite
14.	Oriya	Chireita
15.	Sanskrit	Anaryatikta, Anaryatikta,, Kirāta, Kirātaka, Kirātatiktaka
16.	Tamil	Anariyatittam, Nilavembu, Shirattakuchi
17.	Telugu	Nelavemu
18.	Urdu	Chiraita, Chiaravata , Chiraita shireen

This plant is characterized by its extensively bitter taste, which is attributed to the high quantities of amarogentin, swerchirin and swertiamarin present in this plant. So far, amarogentin is the bitterest compound ever isolated from any plant. These constituents not only give it characteristic bitter taste and aroma, but are also responsible for its medicinal properties [29]. *S. chirata* is having very narrow geographical distribution, limited to higher Himalayas only, from where it has been subjected to over exploitation pushing it towards extinction [14]. Moreover, this plant is very difficult to grow in laboratory as it is having major problems with seed germination and seed viability under laboratory conditions [29,32]. In recent years, *S. chirata* has received significant attention from the scientific community, which has brought the ethnomedicinal importance of this plant to scientific level [33-43].

S. chirata is one of the most important plants used in traditional medicinal system of India. Traditionally, it has been used for the management of variety of ailments as an antihelmintic, carminative, for diarrhea, as a blood purifier, febrifuge, expectorant, antiperiodic and laxative agent, besides, it is known to be beneficial in the management of various skin disorders, malaria, bronchial asthma, gout, etc. [44-46]. Further, this plant has been reported to have central nervous system (CNS) modulatory potential and has been used as a CNS stimulant [47]. Commercially, *S. chirata* is a main ingredient in several formulations (Table 2.2) which includes likes of Ayush-64, Livnol.), Lekorin, Citazil, Malarin, Mahasudarshan churna, Melicon V ointment, Diabecon etc., which indicate its huge medicinal importance.

2.2. OVERVIEW OF THE BOTANICAL DESCRIPTION OF *S. CHIRATA*

S. chirata is a perennial herb which attains the height up 60-160 cm [48]. Upon maturation, stem of this plant measures about 100 cm long, having a diameter of 6 mm. Stem can be identified by its typical appearance. Its stem is glabrous and is somewhat quadrangular on top and is cylindrical in shape at the bottom. It has an appearance form yellowish-brown to purplish, and do possess a large continuous separable yellowish pith all around it [45-49]. The plant bear different types of leaves are vegetative and reproductive stages. During vegetative phase, the plant bears radical leaves and during reproductive phase it bears cauline

leaves. Generally, vegetative phase last for first two years and reproductive phase is completed in third and final year.

Table 2.2: Herbal formulations which has *S. chirata* as a main therapeutic ingredient

S. No.	Manufacturer (s)	Ayurvedic Formulation(s)
1.	Alkem Laboratories Ltd.	Livoerb
2.	Ban Labs Pvt. Ltd.	Livex
3.	Cadila Pharmaceuticals Ltd.	Melicon V ointment
4.	Himalaya Herbal Healthcare	Diabecon
5.	J & J Dechane LaboratoriesPvt. Ltd.	Herbitars, Iobine
6.	Muniyal Ayurveda Pharmaceuticals	Muniliv
7.	SOL Pharmaceuticals Ltd.	Gludip
8.	Standard Pharma Remedies	Biligen
9.	The Central Council for Research in Ayurvedic Sciences, India	Ayush-64
10.	Unexo Laboratories Pvt. Ltd.	Citazil, Lekoril, Lekorin, Livnol, Malarin
11.	Unijules Life Sciences Ltd.	Ayush-64
12	Vhca Ayurveda	Ayush-64
13.	Zandu Ayurveda	Chirakin, Maha sudarshan churna

The radical leaves appear purplish dark-green to light-green to in colour and are having 5 to 7 nerves. Leaves have pinnately reticulate venation and are about 0.24-0.29 m long and about 5.5-6.4 cm wide [48]. With the emergence of main stem, these radical leaves gradually decrease in number and are almost vanish at the onset of floral bud. On the other hand, cauline leaves are broadly ovate, lanciolate, opposite and are sub sessile having acute tip with about 5-7 prominent lateral veins. The length of the cauline leaves varies from 9.7-17.2 cm and the breadth from 3.1-6.8 cm [2,48]. Juvenile cauline leaves have a characteristic dark purplish tinge on the lower lamina which is transformed to a dark green colour upon maturation. This colour change is used to identify the age and maturity of the pant [48].

S. chirata bears branches from the axils of its leaves. These branches undergo further sub-branching giving paniculate type of inflorescence. *S. chirata* bears numerous small green-yellow coloured flowers, which are about 25mm wide, tetramerous, ovoid and are having shades of purplish to white hairs [2, 45]. Seeds of *S. chirata* are very small and are dark brown in appearance. Seeds are irregularly ovoid, approximately 0.16-0.45 mm broad, 0.25-0.55 mm long and angular in outline, while the seed surface characterized by polygonal reticulations[48, 49, 50]. Further, *S. chirata* bears a simple and tapering roots which are short (about 7-8 cm in length and 0.5 inch broad) and yellowish in colour. They are profuse secondary and tertiary roots developed around the collar region of main root [2, 28, 49].

2.3. TRADE AND CONSERVATION STATUS

S. chirata has been used extensively in the traditional system of medicine in the Indian subcontinent and is in high demand in market. Its high medicinal value and increasing demand has made *S. chirata* as one of the largest exported medicinal plant and non-timber forest products (NTFPs) from Nepal [45, 51-55]. *S. chirata* is having broad range of medicinal values and its demand is very high in both international and national market. Further, the demand for *S. chirata* has shown an increase of more than 10% annually in recent years [45, 53]. Globally, Nepal is major exporter of *S. chirata*. Nepal alone account for more than 45% export share of *S. chirata* in the world [45, 52]. In Nepal, there are a total of 17 species which are extensively traded in Nepal, viz. *S. chirata*, *S. bimaculata, S. nervosa, S. dilatata*, *S. minor, S. petiolata, S. angustifolia*, *S. paniculata, S. elegans, S. alata, S. ciliata*, *S. tetragona*, *S. multiflora, S. multicaulis*, *S. lawii, S. densiflora* and *S. racemosa* [45- 55]. Due to the extensive demand of *S. chirata* in global market, it has been subjected to adulterations with the morphologically related species. Adulteration has adversely affected not only its trade, but the clinical applicability as well. Majority of non-gentian adulterants of *S. chirata* include *Andrographis paniculata*, *Exacum spp*., *Ainslia ealatifolia* and *Slevolgia orientalis* [45]. *S. chirata* is having high adulteration rates up to 20%, however, only 5% adulteration is the permissible limit. Uncontrolled adulteration in *S. chirata* has resulted in reduction in its global medicinal value and adversely affected the trade of this herb in both national and international market [46].

2.4. ADULTERANTS AND *S. CHIRATA*

Adulteration is the practice in which original herbal drug with high medicinal value is substituted either completely or partially with the other resembling species to increase the profit. These adulterants are generally inferior to original drug and either lack therapeutic potential or show sub-maximal activity, besides, varying significantly in chemical properties. Adulterants may also be spoiled drug, low grade drug or an entirely different drug which is similar to the original drug, especially morphologically [56, 57]. These adulterated drugs generally do not confine with the established official standards. Moreover, any herbal drug is also considered to be adulterated if it consists any decomposed, putrid or any filthy substance, in part or whole [56, 57]. Adulteration has existed from very long time and has resulted in lowering our faith in traditional medicinal system [58].

Generally, medicinal plants are free from any major adverse effects or show adverse effects of very low grade, and therefore herbal therapy is generally considered safe for use in humans. However, it has been observed that the adverse reports which were reported after the use of herbal drugs, are actually not due to the drug, rather these are the consequences of the added adulterants. To gain extensive profit from the trade of herbal drugs, traders have developed efficient scientific methods for adulterations such that it is very difficult to detect the adulteration and the degree of adulteration with the simple microscopic and chemical analysis. Adulteration result in the compromised patient safety, unpredictable and inconsistent therapeutic response, therefore, the need of the day is to develop some efficient and quick techniques which can easily and accurately detect the degree of adulterations in herbal drugs.

Owning to the high medicinal value of *S. chirata*, it has been extensively exploited from the wild. To meet the market demand, *S. chirata* has attracted several adulterants for gaining major market profit. Till date, the demand of *S. chirata* to fulfill the need of medicinal industry is met by collection from its natural habitat. The extensively increasing demand from the pharmaceutical industry, drastically reduced supply form the wild sources and enormous trading opportunities with *S. chirata* has increased the incidents of adulteration

with spurious varieties and with several plant which resembles morphologically to *S. chirata.* This leads to the reduced therapeutic efficacy of the formulations in which this plant is used. *S. cordata* and *Andrographis paniculata* are two most common adulterant or substitutes of *S. chirata*, and these plants possess poor therapeutic efficacy when compared to *S. chirata* [29, 45]. Further, genus Exacum is another major contributor of the adulterant to *S. chirata.* The plants commonly used as adulterants to *S. chirata* form this genus are *E. xacum tetragonum*, *E. bicolour* and *E. pedunculatum*. Moreover, several different species of genus *Swertia* also are found being used as adulterants which include *S. angustifolia*, *S. cordata, S. ciliata*,

S. bimaculata, *S. elegans*, *S. alata*, *S. paniculata*, *S. minor*, *S. lawii*, *S. densifolia*, and *S. multiflora* [13, 45, 47, 53].There are several methods which have been described and utilized to identify adulterants, most of which are very expensive which limit their regular and general use. This include techniques like morphological and taxonomical investigations [59], analytical processes like Thin Layer Chromatography (TLC), HPTLC, HPLC, gas chromatography, UV/Visible spectrophotometrical analysis, FTIR, NMR, Mass spectroscopy, DNA based techniques (which include RFLP, AFLP, SSR, SNP, STR, RAPD,VNTR, CAPS, LAMP, SCAR, DNA bar coding, Next Generation Sequencing (NGS) etc.) and sophisticated fingerprinting techniques [60]. In past, these techniques has been used to identify the quality and purity of *S. chirata,* however, this is a huge financial burden and result in limited use in practice [59, 61-67].

2.5. PHYTOCHEMICAL PROFILE OF *S. CHIRATA*

S. chirata is has been used for treating several ailments since the beginning of civilization and is currently used in both traditional and modern medicine systems across the globe. In recent year, *S. chirata* has gained lot of attention from the scientific community which has led to the exploration of phytochemical composition of this plant and their medicinal uses. The phytochemical library of *S. chirata* is growing continuously as extensive research on it are identifying new biomolecules regularly, which are being regularly added to the library [35, 68-74]. The therapeutic potential of this plant have been attributed to the wide variety of biomolecules present in it, which mainly include molecules belonging to the class of xanthones, xanthone-derivatives, flavonoids, terpenoids, iridoids and secoiridoid glycosides [75]. Extensive research concludes Swertiamarin, amarogentin and mangiferin as

the major bioactive constituents of *S. chirata* [1, 53]. List of important phytochemical/ biomolecules isolated from *S. chirata* has been summarized in Table 2.3 below.

2.6. MEDICINAL PROPERTIES OF *S. CHIRATA*

2.6.1. ETHNOMEDICINAL VALUE

S. chirata is a well-known and widely used herb in traditional Indian medicinal systems like Ayurveda. In Ayurveda, this plant has been extensively described with medicinal properties viz. Guna :Laghu, Rukaa, Vipaka : Kau, Rasa : Tikta, Virya : Sita, and Karma : Jvaraghna, Kaphapittahara, Raktasodhaka, Vraasodhana, Saraka, Tapaha [1, 29]. Whole plant of *S. chirata* has been used in traditional medicinal systems for the management of ailments and disorders like general weakness, fever, gout, common cold, asthma, cough, joint pain, eczema, vomiting, pimple, stomach disorders, kidney disorders, liver disorders, gastro intestinal tract (GIT) infections, ulcers, hiccups, etc. [1, 29]. The ethnomedicinal use of *S. chirata* has been summarized in Table 2.4 below.

2.6.2. RECENT RESEARCH ON THE MEDICINAL VALUES OF *S. CHIRATA*

2.6.2.1. ANTI-DIABETIC PROPERTIES

A study conducted by Saxena et al. [75] evaluated the effect of *S. chirata* on the diabetes in rats. In this study, they prepared hexane fraction from the whole plant extract and isolated a biomolecule called swerchirin from it. They reported that treating rats with the hexane extract of *S. chirata* (50 mg/kg body weight) resulted in approximately 60% decrease in the hyperglycemia, when measured at 7 hr of administration [75]. The presence of bellidifolin and swerchirin in the *S. chirata* is known to be associated with its antidiabetic potential. These molecules have been reported as potent antidiabetic moieties with a significant dose dependent antidiabetic activity [76, 77]. Further, literature reports that swerchirin, which have been isolated from the hexane extract of *S. chirata*, is having a significant antidiabetic effect on albino rats during fasting condition, non-fasting condition and in glucose tolerance test [78].

Table No. 2.3. List of secondary metabolites present in *S. chirata* (adapted from (Kumar & Van, 2015; Joshi and Dhawan 2007) [1, 29]

S. No.	Compounds	Chemical nature
1	1,3,5,8-tetrahydroxyxanthone	Xanthone
2	1,3,7,8-tetrahydroxyxanthone	Xanthone
3	1,3,6,7-tetrahydroxy xanthone C-2-β-Dglucoside (Mangiferin)	Xanthone
4	1,3,8-trihydroxy-5-methoxyxanthone	Xanthone
5	1,5,8-trihydroxy-3-methoxyxanthone (Bellidifolin)	Xanthone
6	1,7,8-trihydroxy-3- methoxyxanthone (Swertianin	Xanthone
7	1,8-dihydroxy-3,5-dimethoxy xanthone (Swerchirin)	Xanthone
8	1,8-dihydroxy-3,7-dimethoxy xanthone (7-O-methylswertanin)	Xanthone
9	1-Hydroxy-3,5,8-trimethoxyxanthone	Xanthone
10	1-Hydroxy-3,7,8-trimethoxyxanthone	Xanthone
11	2,5-dihydroxyterephthalic acid	Aromatic carboxylic aci
12	21αH-hop- 22(29)-en-3β-ol	Triterpenoid
13	Amarogentin	Seco-iridoid glycoside
14	Amaroswerin	Seco-iridoid glycoside
15	Chiratanin	Dimeric xanthone
16	Chiratenol	Hopane triterpenoid
17	1,5 dihydroxy 3,8-dimethoxyxanthone	Xanthone
18	Decussatin	Xanthone
19	Enicoflavine	Triterpenoid alkaloid
20	Episwertenol	Triterpenoid

S. No.	Compounds	Chemical nature
21	Erythrodiol	Hexane extract
22	Gammacer-16-en-3β-ol	Triterpenoid
23	Gentianine	Triterpenoid alkaloid
24	Gentiocrucine	Triterpenoid alkaloid
25	Gentiopicrin (Gentiopicroside)	Seco-iridoid glycoside
26	Isoorientin	Flavone
27	Kairatenol	Hexane extract
28	Loganic acid	Iridoid
29	Lupeol	Triterepene alcohol
30	Mangostin	Xanthone
31	Oleanolic acid	Triterpenoid
32	Pichierenol	Swertane terpenoid
33	Sweroside	Seco-iridoid glycoside
34	Sweroside 2’-O-3”,3’”,5”-trihydroxybiphenyl-2”-carboxylic acid ester	Seco-iridoid glycoside
35	swerta-7,9(11)-dien-3β-ol	Swertane terpenoid
36	Swertanone	Triterpenoid
37	Swertenol	Triterpenoid
38	Swertiamarin	Seco-iridoid glycoside
39	Taraxerol	Triterepene alcohol
40	Ursoilic acid	Triterpenoid
41	β-Amyrin	Triterepene alcohol
42	β-Sitosterol-3-β-D-glucoside	Sterol
43	Taraxasterol or heterolupeol	Hexane extract

Table 2.4: Ethnomedicinal use of *S. chirata* (adapted from Joshi and Dhawan 2007) [29].

S. No.	Traditional use	Plant part used
1.	As tinctures and infusions in British and American pharmacopeias	Whole Plant
2.	Used in Ayurveda, Siddha and Unani	Whole Plant
3.	Used as effective tonic for general weakness, fever, the common cold, asthma, cough and joint pain Root	Roots
4.	In case of headaches and hypertension, the leaves and stems are finely chopped and soaked overnight in water. The soaked material is ground to a fine paste and filtered. The paste is taken daily and can be consumed in 2-3 days.	Whole Plant
5.	In Tremor fever, the entire plant of Chirata is finely chopped and boiled in 0.5 L of water until the volume is reduced to half. The liquid is filtered and stored in a glass bottle. For children the posology is half a spoon/day for 2 days, and for adults, it is one spoon/day for 2 days or 3 times/day, till the ailment is cured.	Whole Plant
6.	To cure malaria Chirata is boiled in water and a cup of decoction is taken orally.	Whole Plant
7.	In order to cure various skin ailments (such as eczema and pimples) paste of the plant is used	Whole Plant
8.	Stomach, kidney and liver disorders	Whole Plant
9.	Gastrointestinal infections, ulcers, vomiting and hiccups.	Whole Plant
10.	In cases of scorpion bite a combination of Chirata and other drugs is used	Whole Plant
11.	In case of excessive vaginal discharge	Whole Plant

2.6.2.2. ANTICANCER PROPERTIES

Recent literature report both, *S. chirata* extract and isolated biomolecules, to possess significant anti-carcinogenic potential. In one such study, bellidifolin, swertiamarin and their isoforms isolated from the methanolic extract of *S. chirata* were reported to have significant anti-mutagenic activities, when tested against *Salmonella typhimurium* TA100 (S9 mix) [79]. Other literature studies reported the presence of amarogentin and amaroswerin in the methanol extracts of *S. chirata.* These molecules were isolated and tested for their anticarcinogenic potential. It was reported that these molecules were having a significant anti-mutagenic efficacy against *S. typhimurium* [80, 81] evaluated the anti-carcinogenic activity of *S. chirata* and demonstrated the activation of four detoxification enzymes, viz. glutathione, glutathione peroxidase, sulfoxide dismutase and catalase, during cancerous state, after the treatment with *S. chirata* concoction.

2.6.2.3. ANTI-MALARIAL PROPERTIES

A formulation by the name of Ayush-64 is being marketed for anti-malarial activity by The Central Council for Research in Ayurvedic Sciences (CCRAS), India. This formulation contains extracts of *S. chirata*. Another formulation, Malarin, has been developed and marketed by Unexo Laboratories Pvt. Ltd. for the management of Malaria which also contains the extracts of *S. chirata.* Further, Bhat and Surolia[82] reported significant anti-malarial activity of *S. chirata* extract against *Plasmodium falciparum*. A bioactive constituent, swerchirin, was isolated from *S. chirata* and was tested for its anti-malarial activity. Authors reported that swerchirin was having a significant antimicrobial activity against the malarial protozoa [83]. Moreover, a different study also reported the anti-malarial activity of *S. chirata* extracts against *Plasmodium berghei*, when tested on mice [84].

2.6.2.4. HEPATOPROTECTIVE PROPERTIES

Hepatoprotective potential of *S. chirata* has been reported by Hase et al. [85]. This study demonstrated that sweroside isolated from *S. chirata* was having a potent hepatoprotective activity when tested against galactosamine / lipopolysaccharide induced hepatic damage in mice. In another study, methanol extract of *S. chirata* was reported to be a potent hepatoprotective intervention when tested against carbon tetrachloride induced hepatic toxicity [86]. Further, a different study conducted by Nagalekshmi et al. [37] and Verma et al. [87] demonstrated the hepatoprotective effect of a combination of *S. chirata* and *A. paniculata*.

2.6.2.5. ANTI-INFLAMMATORY PROPERTIES

1,5-dihydroxy-3,8-dimethoxyxanthone is a bioactive constituent present in the *S. chirata.* It has been evaluated for its anti-inflammatory potential in rats in a study using carrageenin and formalin-induced pedal edema [88]. The study demonstrated that administered drug at a dose of 50 mg/kg significantly inhibited inflammation by 57% and 58% in carrageen in and formalin-induced inflammation, respectively [88, 89] demonstrated that aqueous extract of the stem of *S. chirata* efficiently modulated the balance between pro-inflammatory and anti-inflammatory cytokines. Their study reported the modulation of interleukin10, interferon, tumor necrosis factor and interleukin1 in a dose dependent manner. This activity was attributed to the presence of amarogentin and mangiferin in the *Swertia* extract. Likewise, Banerjee et al. [88] and Das et al. [21] also demonstrated anti-inflammatory potential of *S. chirata* on various experimental models.

2.6.2.6. ANTIMICROBIAL PROPERTIES

There have been numerous studies conducted on *S. chirata* investigating its antimicrobial properties. The plant has shown activity against both gram positive and gram negative bacteria. In one such study, Laxmi et al. [90] prepared the methanolic and aqueous extract of *S. chirata* and evaluated and demonstrated a potent antimicrobial activity by testing it against 10 bacteria and 3 fungi Nyein et al. [91] also investigated the antimicrobial potential of *S. chirata* against both gram negative and gram positive bacteria. They reported that crude whole plant extract of *S. chirata* is having a significant antimicrobial property against *Bacillus cereus, Salmonella arizonae, Escherichia coli* and *Staphylococcus aureus*.

2.6.2.7. ANTIOXIDANT PROPERTIES

Mahmood et al. [92] evaluated and demonstrated the antioxidant potential of methanolic and aqueous extracts of *S. chirata* on rats. They also demonstrated that plant extract resulted in a significant DPPH radical scavenging. Similarly, Roy et al. [93] investigated the antioxidant potential of methanolic root and leaf extract of *S. chirata* and *S. cordata* along with its antidiabetic and antibacterial potential. They demonstrated that the root extracts of these plants possessed a significant antioxidant activity, however antibacterial and antidiabetic activity was not that much prominent for root extract. However, the methanolic leaf extracts of these plants demonstrated both antibacterial and antidiabetic potential but were lacking significant antioxidant properties. Kumar et al. [94] also demonstrated the antioxidant activity of *S. chirata* by using its aqueous leaf extract [94].

2.6.2.8. ANTI-LEISHMANIAL ACTIVITY

Singha et al. [95] have demonstrated a significant anti-leishmanial activity of *S. chirata* by testing it against *Leishmania donovani* [95]. Bioactive constituents present in the methanolic extract of *S. chirata* such as amaroswerin, amarogentin and sweroside, possess a potent antileishmanial activity [96]. These biomolecules have been demonstrated to inhibit catalytic activity of the protozoan topoisomerase-I and thereby they prevent binary-complex formation. Further, Medda et al. [97] demonstrated anti-leishmanial property of amarogentin a bioactive phytoconstituent of *S. chirata* [97].

2.6.2.9. ANALGESIC AND ANTIPYRETIC PROPERTIES

Bhargava et. al. [98] reported the analgesic and antipyretic potential of *S. chirata* when it was investigated and determined to reduce Brewer's yeast induced pyrexia in albino rats [98]. It also resulted in alleviation of Typhoid-Paratyphoid A, B vaccine induced

hyperexia in rabbits [98]. A study conducted to evaluate the analgesic activities of ethanol extracts of *S. chirata* demonstrated that the extracts inhibited acetic acid induced writhing in mice [99].

2.6.2.10. GASTROPROTECTIVE PROPERTIES

The gastroprotective potential of the ethanolic extracts of *S. chirata* have been demonstrated in rats. Plant extract was demonstrated to significantly improve the damage of gastric mucosainflicted by methacin and necrotizing agents [100]. This study demonstrated that pre-treating rats with ethanolic extract of *S. chirata* prevented ethanol induced gastric mucosal damage and resulted in reestablishment of the non-protein sulfhydryl content in the glandular stomachs.

2.6.2.11. CENTRAL NERVOUS SYSTEM (CNS) MODULATORY ACTIVITY

S. chirata has been demonstrated to possess both CNS stimulating and depressing effect in experimental animals. Literature studies suggest that treating rat and mice with the *S. chirata* extract significantly improved the CNS stimulating effects of mangiferin. This activity was associated with the presence of bioactive phytoconstituent swertiamarin in the plant extract [101].

2.6.2.12. POTENTIAL AS A HAIR TONIC

Apart from the above mentioned use of *S. chirata* in several ailments and disorders, it has also been reported to act as a hair tonic. Swertiamarin, which is a bioactive ingredient of *S. chirata*, has been reported to act as an effective hair tonic. Therefore, it is used in many herbal hair tonics [102].

2.7. OXIDATIVE STRESS

Oxidative stress plays a central role in the development and progression of a variety of complications such as hepatotoxicity, neurotoxicity, diabetes, and Alzheimer's disorder [103-107]. Our body is having an efficient antioxidant defense in the form of catalase, glutathione (GSH), thiols, sulfoxide dismutase, etc., which rapidly neutralize the reactive oxygen and nitrogen species (ROS/RNS) [103, 108]. However, our oxidative stress defense mechanism is a saturable process. When oxidative stress is increases in our body, especially during hypoxia and diabetes, ROS/RNS neutralizing capacity of antioxidant defense is saturated and ROS/RNS enters into the circulation. These free radicals interact with the biomolecules such as proteins, lipids, DNA, RNA and inhibit their normal functioning,

leading to the development of a variety of complications [103, 108, 109]. The liver is responsible for the metabolism and detoxification of a variety of drugs and toxins and therefore, is on the higher risk of the development of oxidative stress-mediated toxicity [110]. Oxidative stress is known to inflict liver toxicity and other liver disorders, such as non-alcoholic steatohepatitis and alcoholic liver disease [110, 111]. Moreover, oxidative stress is markedly enhanced during hypoxic state which further deteriorates the liver functions and is associated with the development and progression of liver complications [112-116]. To date, there is no drug available which can counter oxidative stress and associated complications during hypoxia. There is an urgent need for the screening of some safer and alternative therapeutic strategies which can effectively counter oxidative stress and, therefore, may attenuate associated complications. Plants have long been exploited as a source of safe and efficient therapeutic agents and may provide us with the needed antioxidant therapy which can not only alleviate the oxidative stress but may also halt the progression of ROS and RNS-mediated complications [117, 118]. *S. chirata* has long been used in Ayurvedic and Unani medicine as an antihelminthic, febrifuge, stomach, and liver tonic [1, 119]. This plant is abundant with a large number of alkaloids and flavonoids along with other different phytoconstituents that are responsible for its all broad range of medicinal properties [1,119]. Research work focused on *S. chirata* suggests that plant extract is beneficial in the management of liver disorders; however, the mechanism through which hepatoprotection is achieved remains unknown to a large extent.

CHAPTER 3

INVESTIGATION OF THE USE OF CANDIDATE DNA BARCODES IN DISCRIMINATING *SWERTIA CHIRATA* FROM ITS ADULTERANTS

ABSTRACT

Swertia chirata is a traditional herb widely used in all different pharmacopeias to treat various health ailments. However, high market value and similar morphological characteristics of *Swertia* species, many adulterants of *S. chirata* are available in the market which goes by the name of chirata. In present investigation, differential identification of four adulterant species along with nine *Swertia* species were examined by using six DNA candidate barcodes (rbcL, matK, atpF-H, rpoB, ITS, psbK-I). To test the efficiency of the candidate barcode regions all six inter and intra sequence divergence parameters were calculated. Our results suggest that inter specific divergence was high than intra specific divergence in case of psbK-I when compared to rest of the regions. Also, the single nucleotide polymorphism obtained in psbK-I region and the Neighbor-joining tree obtained by Kimura 2-parameter model, method further supported the potential of the region to be used as barcode to identify *S. chirata* and differentiate it from most commonly used adulterants. In conclusion, barcode psbk-I can be used proficiently to identify *S. chirata* and its adulterants, and it can also provide useful information regarding quality evaluation, sustainable utilization and resource protection of this medicinal plant.

3.1 INTRODUCTION

The demand of *S. chirata* in pharmaceutical industry ever increasing is very high and it has been extensively exploited from its wild habitat. Since roots are most potent part of the plant so plant need to be uprooted for commercial used. Destructive extraction and over exploitation of the plant from natural habitats have resulted in drastic reduction of natural populations and the figures in list of endangered species. Drastically reduced supply form the wild sources and enormous trading opportunities with *S. chirata* has increased the incidents of adulteration with spurious plant species which resembles morphologically to *S. chirata*. Increased concern are being raised about safety and efficacy of herbal products, which were once considered as safe and free from side effects due this menace of adulteration [16, 120].Adverse consequences of species adulteration on the health and safety of consumers have also been reported recently [121]. *Andrographis paniculata* and other *Swertia* species such as *S. angustifolia, S. paniculata, S. ciliate* and *S. cordata,* are often misused or used as substitutes of *S. chirata* and thus affects the trade and economics of *S. chirata* [5].

Although, *S. chirata* getting high attention from the wide range of researchers as marked number of publications appear in literature. But for authentication of *S. chirata* few criteria and certain traditional methods have been developed which are mainly based on genetic phenotypes and analysis of chemical components [18]. All such available methods used are affected by some intrinsic and extrinsic factors such as time of harvest, availability of experts and processing methods etc. Whereas, morphological identification becomes problematic due to very close resemblance of substitute species and when original plant material in herbal formulations is grinded or used in any non-recognizable form. Therefore, keeping in view the current medicinal status of *S. chirata,* its scarcity and problem of adulteration, there is an urgent need a robust method of species identification which not affected by environmental and other factors and easy to apply on even processed plant material. The present study is an attempt to evaluate some of the highly recommended plant DNA bar coding loci to determine their efficiency to be used for the identification and authentication of *S. chirata* from its adulterants.

In recent years, a new diagnostic technique known as DNA barcodes has evolved which can be used as an efficient tool for the species identification [122]. This approach includes sequencing of short standard DNA region from a small fragment of genome, which can be amplified by universal primers [18]. Consortium for the Barcode of Life, Plant

Working Group recommended several regions that can be used as candidate barcodes for plants. Some are non-coding spacers of coding gene such as Megakaryocyte-Associated Tyrosine Kinase (mat K), ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rcbL), beta subunit of RNA polymerase (rpoB) and DNA-dependent RNA polymerase (rpoC1)[18,19].Whereas, other are multi-region combinations such as combinations of rbcL and matK, trnH-psbA+ rbcL etc.

In this investigation, six barcodes (ITS, psbA-trnH, matK, rbcL) from plastid and nuclear genome were evaluated to authenticate *S. chirata* and related species used as adulterants. The primary aim of investigation was to develop species identification method for *S. chirata* which can discriminate *S. chirata* from its adulterant species.

3.2 MATERIAL & METHODS

3.2.1 PLANT MATERIAL

Plant specimens, which belonged to 6-different species of *Swertia* (Family: Gentianaceae) were used. These samples or specimens were collected in the months between July and September (2011) from the tropical Himalayan regions of Himachal Pradesh and Uttarakhand. The maps of states Himachal Pradesh and Uttrakhand, locations where plant sample were collected are depicted in Figure 3.1 and Table 3.1 respectively. *Picrorhiza kurroa* maintained in Department of Biotechnology, Jaypee University of IT Solan, and green house was also uses as a sample in the study. The species were got identified and authenticated by Prof. Narain Singh Chauhan (Dr. Yashwant Singh Parmar University Solan, HP).

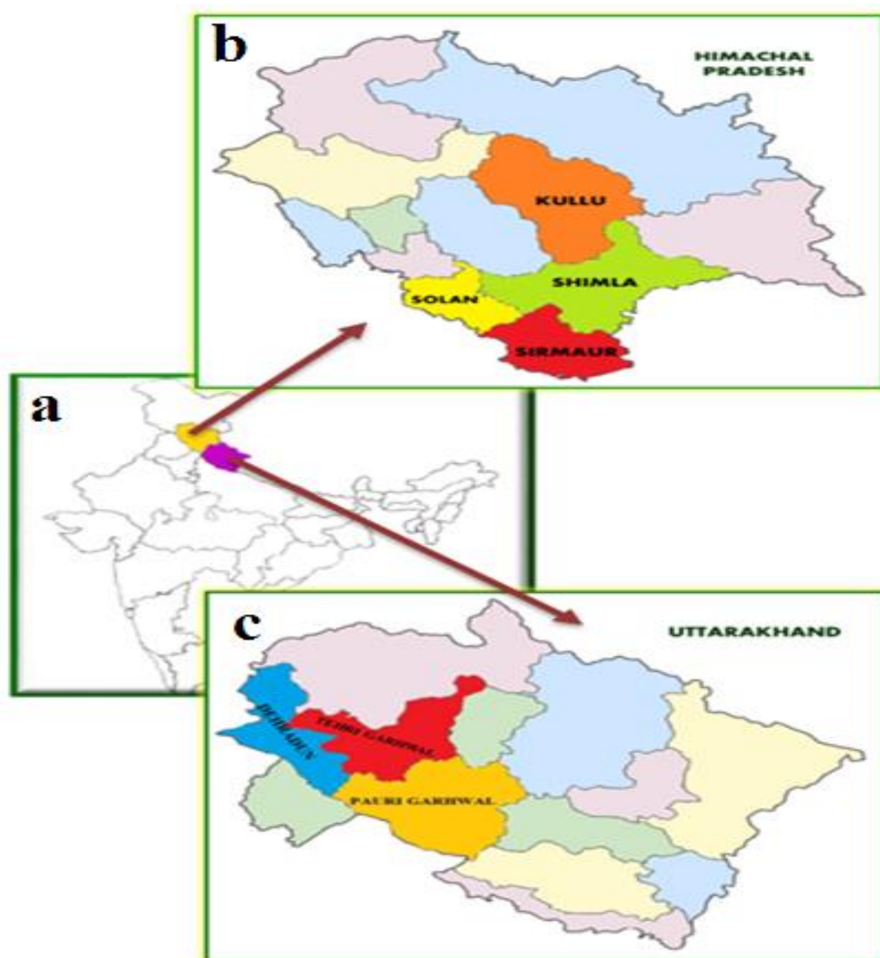


Figure 3.1: Map of Himachal Pradesh and Uttarakhand and from where *S. chirata* and other plant were collected [27, 28]

Table: 3.1: Sampling localities and number of samples collected from each location

Species	Collection Site	No. of samples	Species	Collection Site	No. of samples
Swertia chirata	Nauni (shilly)	4	*Swertia cordata*	Haripurdhar	3
Swertia chirata	Dehradun	6	*Swertia cordata*	Uttrakhand	5
Swertia chirata	Uttrakhand	2	*Swertia ciliate*	Manali	12
Swertia ciliata	Rohru	33	*Swertia ciliate*	Rohru	10
Swertia ciliata	Haripurdhar	5	*Swertia cordata*	Rohru	4
Swertia cordata	Haripurdhar	3	*Swertia angustifolia*	Choordhar	7
Swertia cordata	Rohru	8	*Swertia paniculata*	Choordhar	9
Swertia ciliata	Haripurdhar	5			

3.2.2 DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

The genomic DNA of all the herbal plant material was extracted by using Pure Link Plant Total DNA Purification Kit (Invitrogen) following the given protocol. The quality of isolated DNA was checked by performing electrophoresis using 1% agarose. The primers used to amplify PCR products from candidate DNA barcode regions, are depicted in table 3.2. The PCR reactions were conducted in a final volume of 25 µl containing 9.5 µl 2XTaq MasterMiX (CWBIO), 1.5 µl DNA, 12.5 µl dd H_2O, 1 µl primer using a GeneAmp 9700 thermocycler. The amplification condition consisted of pre denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. But the primer annealing temperature was optimized for every primer pair for obtaining clear and distinct band of PCR product. PCR products were examined agarose gel electrophoresis (2% gel) in 1xTAE buffer at 100 v for 45min.The selected PCR products got sequenced from Xcelris Labs. Ltd. Premchand Nagar Road, Bodakdev, Ahmedabad, India.

3.2.3 SEQUENCE ALIGNMENT AND DATA ANALYSIS

The sequences, of PCR amplified products, were assembled and aligned by utilizing CLUSTALX program, which were then adjusted manually in Bio Edit software (version 7.0.5). Sequence data obtained for all candidate regions of all the species were submitted in Gene bank Nucleotide database. Table 3.2 represents the assigned accession numbers to the sequences obtained.

The data analysis was done for all the genteianace sequences to evaluate the feasibility of candidate regions that can be used as barcode for the identification of species used in the study. The nucleotide diversity was examined of all the candidate DNA regions among all the species with the help of DNAsp software. The sequences were aligned with the help of Clustal Omega software. Further, MEGA5.0 was used for calculating the genetic distances according to Kimura 2-parameter (K2P). The candidate regions were evaluated based upon the genetic divergence calculated using six parameters. The six parameters were used to calculate interspecific divergence and intraspecific variability. To characterize inter-specific divergence three parameters were used, (1) average of the interspecific distance observed in each genus with a minimum of at least two species, (2) mean pair wise distance

i.e. theta prime within each genus and, (3) minimum interspecific distance calculated within each genus. Further to calculate intra-specific variation additional three parameters were determined: (1) average intra-specific distance within each species, (2) mean pairwise distance within each species, (3) coalescent depth i.e. maximum intra-specific distance within each species. Interspecific and intraspecific divergence was compared to evaluate the barcoding gap. In addition to this sequence data was analyzed and compared to evaluate the potential of barcode regions in discriminating different species with the help of few most recommended methods (neighbor joining tree based on K2P distance and unweighted pair group method with arithmetic mean).

3.3 RESULTS

3.3.1. AMPLIFICATION AND SEQUENCING OF PURIFIED AMPLICONS

The PCR conditions were optimized for primer annealing temperature of all the candidate regions. The standardized primer annealing temperature amplification of target candidate regions and size of amlicons obtained are given in table 3.2. The annealing temperature ranges from 51°C - 56°C and size of amplicon obtained varied from 300 bp to 862 bp. Successful PCR amplification was obtained using universal primers in most of the candidate barcode regions of all species. The representative gel pictures of amplified PCR products are shown in figure 3.2, 3.3 and 3.4.

After successful amplification the purified amplicons were sent for sequencing. The sequencing data obtained were analyzed further and sequences were submitted to NCBI Nucleotide database. The accession numbers submitted to NCBI and corresponding species are shown in Table 3.3.

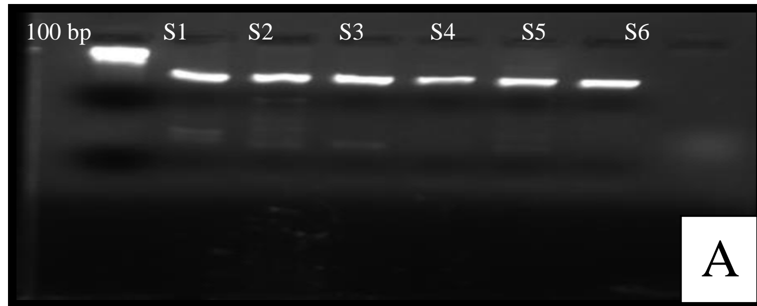
The amplification success rate was maximum with psbk-psbI region followed byITS1-ITS2, atpF-H, rbcl, matK , rpoC, rpoB. The size of amplicons obtained were more or less equal in all the species barring slight variation observed in case of psbK-psbI region as shown in figure 3.4.

Table 3.2: Primer sequences, optimized annealing temperature and size of amplicon obtained w.r.t. candidate regions

Marker	Primer Sequence (5'-3')	Amplicon size	Ta Optimum
RbcL	F: GTAAAATCAAGTCCACCACG	580 b.p.	56°C
	R: ATGTCACCACAAACAGAGACTAAAGC		
psbK-psbI	F: TTAGCATTTGTTTGGCAAG	544 b.p.	51°C
	R: AAAGTTTGAGAGTAAGCAT		
rpoC1	F: GGAAAAGAGGGAAGATTCCG	509 b.p.	56°C
	R: CAATTAGCATATCTTGAGTTGG		
trnH-psbA	F: GTTATGCACGAACGTAATGCTC	300 b.p.	55°C
	R: CGCGCGTGGTGGATTCACAATCC		
RpoB	F: ATGCAGCGTCAAGCAGTTCC	406 b.p.	55°C
	R: TCGGATGTGAAAAGAAGTATA		
atpF-atpH	F: ACTCGCACACACTCCCTTTCC	675 b.p.	53°C
	R: GCTTTTATGGAAGCTTTAACAAT		
MatK	F: CGTACTGTACTTTTATGTTTACGAG	862 b.p.	55°C
	R: ATCCGGTCCATCTAGAAATATTGGTTC		

Table 3.3: Accession Numbers of Sequences of candidate regions of species submitted in NCBI, Nucleotide database

Accession no.	Species	Accession no.	Species
KC594650	*Aconitum heterophyllum*	KC594657	*Picrorhiza kurroa*
KC594655	*Andrographis paniculata*	KC594652	*Andrographis paniculata*
KC594666	*Swertia chirata*	KC594668	*Swertia cordata*
KC594671	*Swertia cordata*	KC594651	*Aconitum heterophyllum*
KC594677	*Swertia ciliata*	KC594656	*Andrographis paniculata*
KC594660	*Picrorhiza kurroa*	KC594667	*Swertia chirata*
KC594653	*Andrographis paniculata*	KC594672	*Swertia cordata*
KC594664	*Swertia chirata*	KC594661	*Picrorhiza kurroa*
KC594669	*Swertia cordata*	KC594649	*Aconitum heterophyllum*
KC594675	*Swertia ciliata*	KC594665	*Swertia chirata*
KC594658	*Picrorhiza kurroa*	KC594676	*Swerita ciliata*
KC594653	*Andrographis paniculata*	KC594649	*Aconitum heterophyllum*
KC594664	*Swertia chirata*	KC594670	*Swertia cordata*
KC594648	*Aconitum heterophyllum*	KC594659	*Picrorhiza kurroa*
KC594663	*Swertia chirata*	KC594670	*Swertia cordata*
KC594674	*Swerita ciliata*		



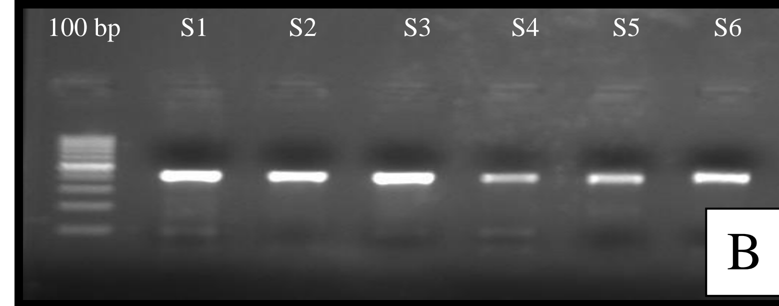
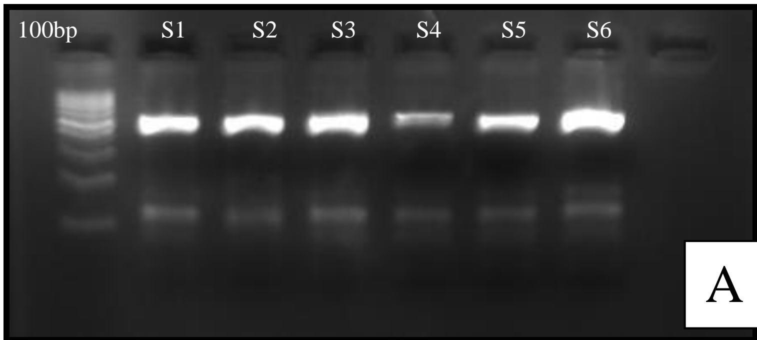


Fig 3.2:PCR amplification Product obtained in case of target region A) rpoB; B) rpo C



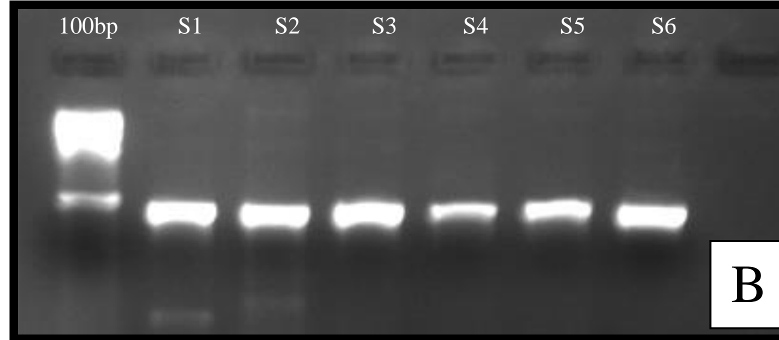
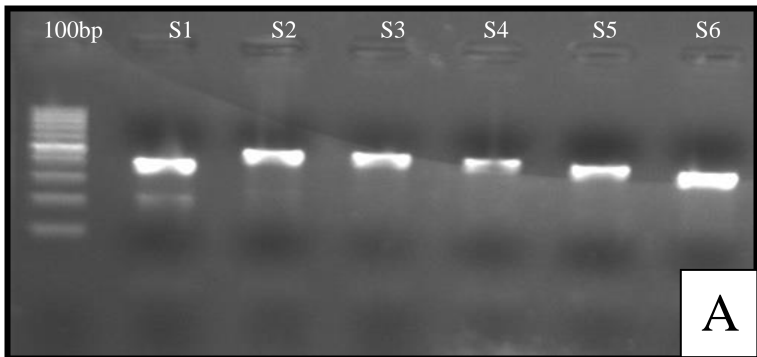


Fig 3.3:PCR amplification Product obtained in case of target region A) rbcl; B) Mat K



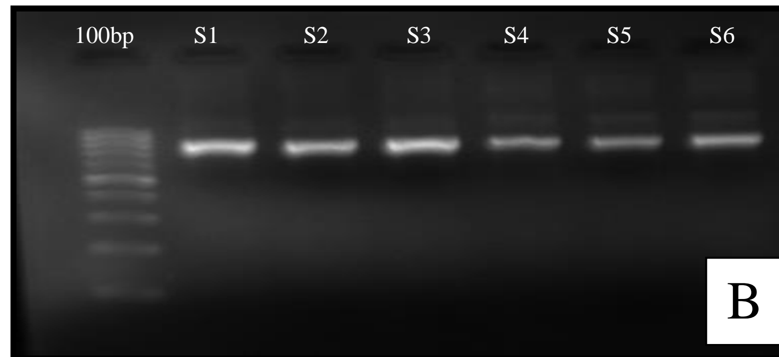


Figure 3.4: PCR amplification Product obtained in case of target region A) psbK-psbI; B) psbA-trnH

3.3.2. NUCLEOTIDE POLYMORPHISM AND PRIMER PERFORMANCE

For all the candidate regions genetic divergence within and between species was calculated using the Kimura 2-parameter model, which is accepted as best model for analysis at significant DNA polymorphism among the sequences of various species using DNAsp software species level with low distances. Significant Nucleotide polymorphism was obtained in case of psbk-I region as shown in Table 3.4 which can be further targeted to discriminate the species from rest of its adulterant species. It was confirmed from results obtained by using primerplex as shown in Table 3.5 which gave the set of primers that can be used to target the nucleotide divergence of psbK-I region.

Table 3.4: Nucleotide polymorphism obtained in PsbK-I target region

PSBK-I (Forward)	83	109	210	399	424
S. Ciliata	a	g	c	c	g
S. Paniculata	a	g	c	c	g
S. Angustifolia	a	g	c	c	g
S. Cordata	c	g	g	c	g
S. chirata	a	a	c	c	a
A. Paniculata	a	g	c	t	g

3.3.3. *IN SILICO* ANALYSIS TO CALCULATE INTER SPECIFIC AND INTRA SPECIFIC DIVERGENCE

All the three inter-specific divergence parameters were compared to investigate the utility of all the candidate DNA regions to be used for the identification of S*wertia* species and its adulterants. To discriminate species an ideal barcode region acquires higher inter specific divergence in comparison to intra specific divergence. Six different metrics were used to differentiate between intra-specific and inter-specific variation as a measure of genetic divergence for each locus. Psbk-I and atpf-H both showed significantly high level of inter-specific discriminatory capability, when compared to the ITS, psbA-trnH, matK, rbcLandrpoB. Further, rbcL demonstrated the lowest divergence amongst specific individuals, when observed in all inter-specific calculations. Furthermore, the results obtained from intraspecific outcomes were comparable, where rbcL demonstrated the lowest variations and atpf-H demonstrated the highest variations (Figure 3.5).

Table 3.5: Output of Primer Plex to target the nucleotide polymorphism of psbK-I region

Accession Number	Sequence definition	Sense Type	Oligo Type	Quality	Rating	Probe Sequence	Position
*000001 [A/G: 553]	IT7	Sense	Wild	Good	74.6	AACTAAGGTGCTGTCGCA	536
*000001 [A/G: 553]	IT7	Sense	Mutant	Good	63.2	AACTAAGGTGCTGTCGCG	536
*000003 [T/G: 170]	IT8	Sense	Wild	Good	74.7	AAGGAAAACAAGAAAGGATT	150
*000003 [T/G: 170]	IT8	Sense	Mutant	Best	75	AAGGAAAACAAGAAAGGATG	150
*000004 [T/C: 204]	IT3	Sense	Wild	Poor	37	TCCGTTCGCGGAGTGCAT	187
*000004 [T/C: 204]	IT3	Sense	Mutant	Poor	28	TCCGTTCGCGGAGTGCAC	187
*000002 [A/G: 492]	No Wild ASPE primer found: 10 rejected (Dimer: 10)						
*000004 [T/C: 83]	No Wild ASPE primer found: 10 rejected (Dimer: 10)						

Accession Number	Sequence Definition	Sense Type	Oligo Type	Quality	Rating	Probe Sequence	Position
*000001 [A/G: 553]	IT7	Sense	Wild	Good	69.1	CAACTAAGGTGCTGTCGCA	535
*000001 [A/G: 553]	IT7	Sense	Mutant	Good	57.7	CAACTAAGGTGCTGTCGCG	535
*000003 [T/G: 170]	IT8	Sense	Wild	Good	74.2	CAAGGAAAACAAGAAAGGATT	149
*000003 [T/G: 170]	IT8	Sense	Mutant	Good	72.8	CAAGGAAAACAAGAAAGGATG	149
*000002 [A/G: 492]	No Wild ASPE primer found: 10 rejected (Dimer: 10)						
*000004 [T/C: 204]	Failed in multiplexing						
*000004 [T/C: 83]	No Wild ASPE primer found: 10 rejected (Dimer: 10)						

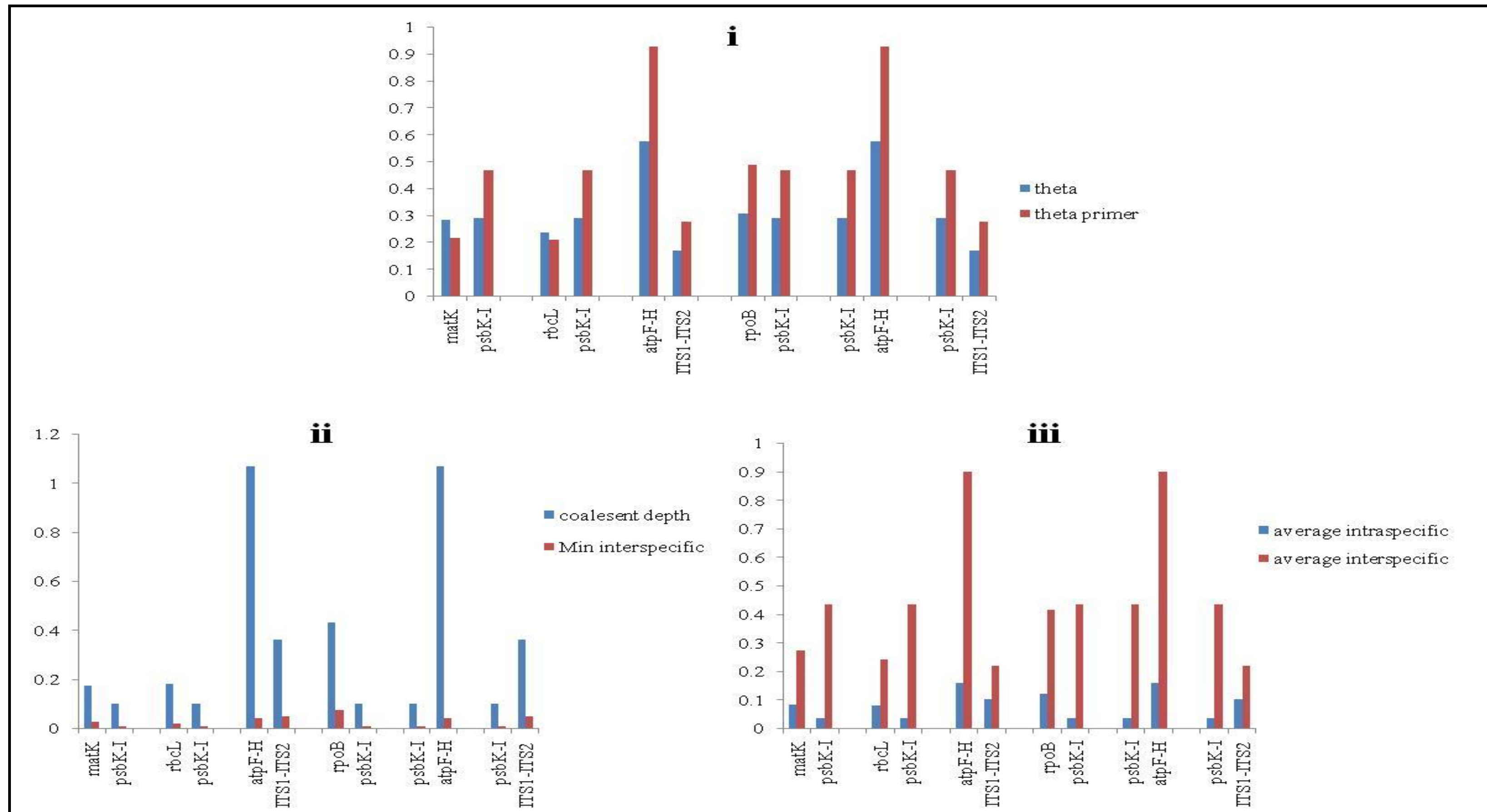


Figure 3.5: Analyses of the inter-specific divergence between congeneric species and intra-specific variation of the six loci. First, three parameters were used to characterize inter-specific divergence: (i) average inter-specific distance (K2P distance) between all species in each genus with at least two species; (ii) average theta prime (θ'), where theta prime is the mean pairwise distance within each genus with more than one species, thus eliminating biases associated with different numbers of species among genera; and (iii) smallest inter-specific distance, i.e., the minimum inter-specific distance within each genus with at least two species. Second, three additional parameters were used to determine intraspecific variation: (i) average intra-specific difference (K2P distance), that between all samples collected within each species with more than one individual; (ii) theta (θ), where theta is the mean pairwise distance within each species with at least two representatives; θ eliminates biases associated with unequal sampling among a species; and (iii) average coalescent depth, which is the maximum intra-specific distance within each species with at least two individuals.

3.3.4. ESTIMATION OF EVOLUTIONARY DIVERGENCE

It should be noted that a constructive barcode must acquire inter-specific divergence on a higher side so as to differentiate different species. Usually, bar-coding study is used for the construction of phylogenetic trees so as to find out species names. Neighbor-joining tree is commonly used method. In this method the estimation is based on phenetic distance and the evolutionary information of the species [123]. In our study, when we analyzed the neighbor-joining tree, we observed that barcode marker of psbK-I effectively differentiated each individual genus by depicting monophyletic clades, such that the each clade appeared completely different from other observed clades as shown in Fig3.6.

Further distance matrix was calculated for psbK-I region to estimate the evolutionary divergence in sequence of different species using the Kimura 2-parameter model. The number of base substitutions per site between sequences is shown in table 3.6 whereas corresponding standard errors are given in other half of the table i.e. above the diagonal. This analysis involved 28 nucleotide sequences. All ambiguous positions were removed for each sequence pair and total of 1060 positions were there in the final dataset. Evolutionary analyses was done using MEGA5 [124].

Table 3.6: Estimation of evolutionary divergence in sequences of psbK-I region.

P46		0.025	0.037	0.025	0.034	0.036	0.027	0.027	0.039	0.041	0.037	0.041	0.029	0.039	0.042	0.039	0.038	0.037	0.042	0.038	0.036	0.029	0.094	0.092	0.108	0.120	0.112	0.114
D46	0.360		0.033	0.024	0.033	0.033	0.028	0.028	0.033	0.043	0.036	0.042	0.029	0.037	0.039	0.039	0.037	0.033	0.040	0.036	0.032	0.027	0.089	0.090	0.108	0.122	0.118	0.111
KI-3	0.587	0.497		0.012	0.026	0.028	0.017	0.018	0.031	0.036	0.031	0.041	0.025	0.040	0.039	0.038	0.038	0.030	0.039	0.034	0.034	0.023	0.086	0.080	0.100	0.130	0.092	0.103
KI-32	0.253	0.246	0.078		0.021	0.020	0.017	0.017	0.023	0.022	0.022	0.023	0.022	0.025	0.025	0.026	0.025	0.020	0.024	0.023	0.024	0.022	0.076	0.075	0.101	0.129	0.095	0.104
KI-8	0.458	0.400	0.314	0.213		0.019	0.015	0.014	0.036	0.034	0.034	0.032	0.027	0.037	0.036	0.035	0.038	0.034	0.033	0.033	0.032	0.027	0.085	0.084	0.114	0.122	0.099	0.106
KI-4	0.583	0.502	0.414	0.197	0.220		0.013	0.015	0.034	0.040	0.036	0.036	0.029	0.040	0.043	0.041	0.038	0.032	0.042	0.035	0.032	0.026	0.088	0.087	0.079	0.119	0.113	0.097
KI-42	0.280	0.292	0.141	0.126	0.103	0.088		0.010	0.026	0.026	0.027	0.028	0.029	0.030	0.029	0.030	0.029	0.025	0.028	0.025	0.027	0.026	0.090	0.088	0.106	0.117	0.100	0.105
KI-82	0.286	0.298	0.161	0.144	0.094	0.107	0.045		0.026	0.024	0.027	0.025	0.026	0.029	0.029	0.031	0.029	0.026	0.027	0.027	0.025	0.024	0.091	0.087	0.112	0.114	0.101	0.105
D41	0.503	0.409	0.364	0.192	0.436	0.435	0.256	0.239		0.029	0.029	0.030	0.023	0.027	0.030	0.028	0.027	0.018	0.027	0.028	0.027	0.020	0.091	0.083	0.106	0.105	0.097	0.112
P45	0.653	0.647	0.603	0.212	0.461	0.615	0.264	0.245	0.351		0.022	0.030	0.017	0.030	0.030	0.030	0.030	0.030	0.029	0.030	0.026	0.019	0.090	0.073	0.103	0.105	0.090	0.107
D45	0.491	0.458	0.391	0.186	0.491	0.499	0.273	0.276	0.355	0.230		0.023	0.015	0.029	0.030	0.027	0.028	0.030	0.027	0.027	0.025	0.016	0.087	0.076	0.106	0.102	0.095	0.106
KI-5	0.695	0.658	0.645	0.220	0.434	0.614	0.291	0.267	0.357	0.439	0.249		0.011	0.032	0.034	0.030	0.031	0.028	0.034	0.028	0.025	0.015	0.093	0.074	0.097	0.097	0.090	0.110
KI-52	0.317	0.316	0.231	0.198	0.303	0.330	0.298	0.264	0.194	0.125	0.099	0.068		0.022	0.020	0.021	0.019	0.018	0.015	0.018	0.016	0.015	0.078	0.070	0.083	0.098	0.087	0.111
P42	0.575	0.501	0.564	0.223	0.495	0.589	0.289	0.281	0.329	0.405	0.340	0.445	0.196		0.022	0.022	0.023	0.026	0.030	0.029	0.027	0.019	0.090	0.073	0.097	0.102	0.108	0.108
D42	0.680	0.594	0.608	0.214	0.477	0.639	0.283	0.279	0.375	0.473	0.362	0.555	0.166	0.277		0.024	0.027	0.027	0.028	0.030	0.025	0.018	0.086	0.072	0.092	0.100	0.097	0.105
P43	0.570	0.525	0.538	0.240	0.456	0.574	0.295	0.312	0.356	0.399	0.317	0.426	0.186	0.263	0.312		0.021	0.027	0.029	0.029	0.023	0.019	0.088	0.074	0.102	0.107	0.101	0.109
D43	0.550	0.488	0.520	0.240	0.485	0.524	0.296	0.299	0.325	0.411	0.366	0.442	0.162	0.303	0.343	0.234		0.024	0.028	0.026	0.021	0.016	0.086	0.077	0.083	0.098	0.111	0.114
P41	0.481	0.397	0.346	0.175	0.440	0.419	0.252	0.255	0.167	0.381	0.349	0.312	0.146	0.297	0.315	0.311	0.264		0.024	0.022	0.023	0.014	0.091	0.081	0.092	0.097	0.090	0.112
D44	0.699	0.641	0.598	0.234	0.429	0.649	0.296	0.275	0.305	0.437	0.337	0.493	0.106	0.426	0.413	0.388	0.364	0.256		0.026	0.019	0.010	0.074	0.073	0.086	0.093	0.085	0.104
KI-7	0.575	0.484	0.457	0.198	0.443	0.518	0.252	0.278	0.333	0.419	0.344	0.381	0.147	0.412	0.397	0.376	0.319	0.244	0.317		0.022	0.011	0.085	0.078	0.095	0.099	0.091	0.102
P44	0.522	0.440	0.444	0.228	0.410	0.446	0.281	0.259	0.298	0.306	0.292	0.295	0.114	0.333	0.309	0.272	0.252	0.243	0.192	0.248		0.010	0.081	0.074	0.091	0.094	0.087	0.105
KI-72	0.289	0.267	0.193	0.186	0.303	0.287	0.266	0.254	0.154	0.149	0.113	0.113	0.102	0.150	0.135	0.147	0.124	0.088	0.046	0.063	0.041		0.076	0.072	0.079	0.090	0.084	0.106
KI-1	1.124	1.083	1.021	0.835	1.052	1.084	0.982	0.990	1.012	1.069	1.034	1.096	0.861	1.042	1.014	1.024	1.011	1.004	0.944	1.000	0.993	0.834		0.016	0.068	0.092	0.078	0.083
KI-12	0.985	0.966	0.886	0.823	0.926	0.973	0.933	0.938	0.875	0.807	0.839	0.828	0.795	0.807	0.796	0.799	0.800	0.873	0.803	0.843	0.810	0.781	0.121		0.077	0.088	0.068	0.080
KI-2	1.331	1.305	1.247	1.069	1.237	1.141	1.082	1.143	1.157	1.294	1.228	1.271	0.944	1.258	1.254	1.252	1.125	1.088	1.192	1.197	1.132	0.906	0.933	0.922		0.014	0.043	0.041
KI-22	1.079	1.087	1.142	1.144	1.114	1.112	1.094	1.087	0.953	0.980	0.975	0.946	0.956	0.963	0.952	0.985	0.938	0.925	0.914	0.943	0.925	0.869	0.951	0.935	0.090		0.041	0.042
KI-6	1.209	1.245	1.071	0.971	1.127	1.221	1.052	1.056	1.109	1.040	1.109	1.045	0.915	1.120	1.062	1.129	1.134	1.016	0.986	1.045	0.976	0.883	0.981	0.791	0.615	0.423		0.015
KI-62	1.044	1.034	1.009	1.012	1.022	0.969	1.007	1.018	1.052	0.971	0.986	1.021	1.032	0.990	0.970	1.033	1.043	1.058	0.995	0.985	1.005	1.001	0.903	0.865	0.460	0.469	0.092	

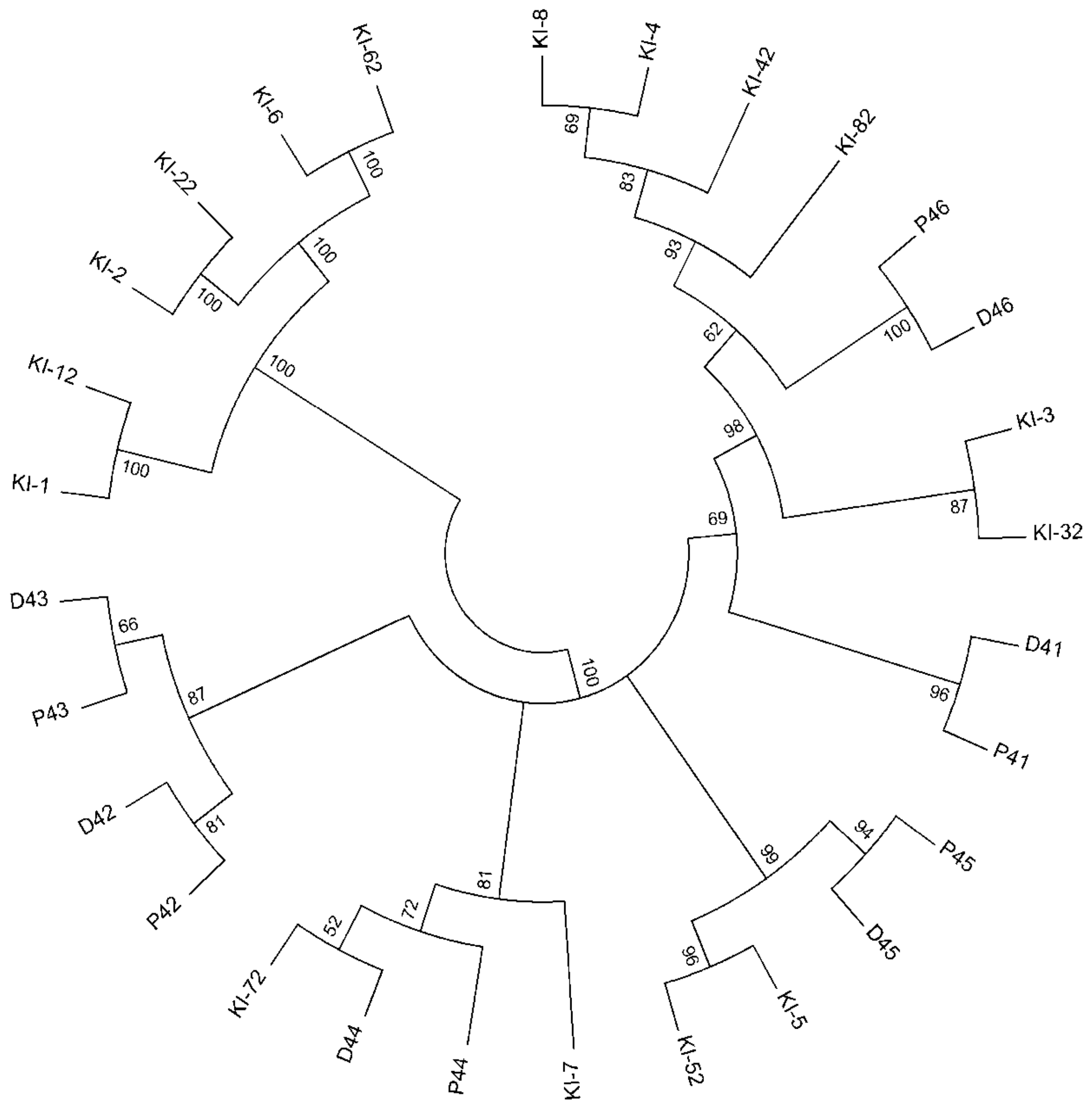


Figure 3.6: K2P distance NJ tree for psbK-I. A consensus NJ tree for *S. chirata* and its adulterants assessed with 1000 bootstrap replicates was constructed by bootstrap analyses with the bootstrap value indicated at the branches (bootstrap values of less than 50 are not shown).

3.4. DISCUSSION

To overcome the difficulties of traditional taxonomy and the prevailing genome-based approaches, [19] proposed DNA bar coding which successfully used in identification, diversity and discovery of unknown species [125]. DNA barcoding has come up as one of the emerging and efficient tool that can be used to solve various complex problems of raw plant material authentication and establish an assurance for quality level in the herbal drug industry [126]. In this technique, consistent region of DNA, a small fragment (<1000 bp) of genome which can be obtained easily is used for detection and is known as DNA Barcode. There are two desirable criteria which DNA barcode should possess. Firstly, the DNA barcode should be having sufficient sequence variations for the identification of the species, and secondly, DNA barcode should be having adequate conserved ranking sites for the depiction of several parameters which are universal. Various species have been used as adulterants of *S. chirata* and are being traded in market as mentioned in literature [18, 127, 128]. It is a first attempt for utilizing standard barcode regions suggested by the CBOL Plant Working Group in 2009 and various other scientists and their groups [18, 128] i.e. Psbk-I, ITS1-ITS2,atpf-H, rbcL, matK and psbA-trnH used for authentication of *S. chirata* and its adulterant species. Genetic divergence within and between species was assessed using various metrices after PCR amplication obtained with the use of universal primers. The main criteria for any region to be used as suitable barcode is that, it must exhibit high interspecific but low intraspecific divergence. Further, atpF-atpH and rbcL are the universal DNA barcodes and are easy to amplify [129]. Further, the rbcl fail to identify all the species, but it widely accepted in differentiating genera as reported by New master et al. [130] in 2008.Although atpF-atpH and matK in combination have been used successfully [128], but in case of *Swertia* species the present results demonstrated that rbcl and matK possessed lower variations in the sequence and therefore, it could not be utilized when *Swertia* species are under investigation. Our findings are in line with the previous reports of Newmaster et al. (2006) [130] where atpF-atpH did not demonstrated any significant variations amongst closely-related species. Therefore, it becomes evident that these markers may only be used along with other barcodes in combination for achieving efficient outcome. Further, there are several other studies which depicts that psbA-trnH could be potential barcodes. In one such finding reported by Kress et al. [127] in 2007, psbA-trnH was observed to efficiently discriminate mosses, gymnosperms, algae, angiosperms and ferns [127, 131]. In the present study, we encountered a lot of difficulties in sequencing of psbA-trnH region, which may probably be due to the presence of

a long poly-A domain in psbA-trnH region, and therefore, reduces the applicability of this experiment. In the current study we compared 6-barcode markers in the discrimination of *S. chirata* from its adulterants, psbK-I was the best option showing maximum inter specific divergence with minimum intra specific divergence psbK-I region was utilized for the construction of the NJ tree, based on the K2P distance bootstrap analysis, was conducted to estimate the statistical supports to the constructed tree.

A desirable and an efficient DNA barcode must be possessing simultaneously, firstly, sufficient variations in the sequences for the identification of different species and, secondly, it must possess suitable conserved flanking sites for the designing universal primers to amplify PCR products. This study indicates that DNA barcodes may also be an efficient tool for the validation and authentication of other medicinal plants as well.

3.5. CONCLUSION

Our findings suggest that psbK-I locus can be efficiently exploited for differentiating various *Swertia* species and their adulterants. Moreover, we have demonstrated that the DNA bar coding may prove to be very important tool for authentication, validation, identification and classification of different species. Based on the outcome of our study, we further suggest that DNA barcoding technology may be applied for resolving complicated issues regarding identification and discrimination problems associated with *Swertia* species and their adulterants. However, to be more precise and specific, to improve the identification accuracy, the DNA barcodes may be used in combination with some genus which may be difficult to identify using single barcode. However, in the present study Psbk-I is proposed to be used as a complementary barcode for differentiating *Swertia* species.

CHAPTER 4

COMPARATIVE METABOLOMICS REVEALS THE METABOLIC VARIATION BETWEEN *SWERTIA CHIRATA* AND ITS ADULTERANTS

ABSTRACT

Plants produce variety of secondary metabolites, such as glycosides, alkaloids, tannins, saponins etc., which possess variety of pharmacological activities and have been used for the treatment of various ailments since ages. Medicinal plants belonging to same family or some genus exhibit remarkable resemblance in their physical properties, and to some extant in their medicinal values. In recent times, there have been remarkable advancement in the techniques like, Liquid chromatography (LC), Gas-chromatography (GC),Mass spectroscopy (MS), GC-MS, LC-MS etc., which has made it feasible to separate the individual components of herbal material, identify it, analyze it and differentiate various herbal preparations in the basis of received outcome. In the present study the adulterant species of *S. chirata* were analyzed to identify the metabolites that can differentiate and discriminate the same from its adulterants. The analysis is based on the fact that genotypes of different plant material differs and possess peculiar metabolic profile. The results indicate that Tetradecanal, Methyl 3-hydroxy-2-oxobutanoate, 1,2-benzenedicarboxylic acid are the most potential marker compounds to differentiate *S. chirata* from its adulterants. However a separate detailed study is required to standardize workable protocol. Phylogenetic analysis based on GC-MS generated metablomic data revealed that *S. cordata* is more closely related to *S. chirata* in comparison to *Andrographis paniculata.* In PCA analysis samples with same metabolomic compositions were clustered together and are represented as a separate cluster, while those with different metabolic components were shown as dispersed. By utilizing these approaches, it is possible to efficiently identify not only the presence of adulterants in the herbal preparations of *S. chirata,* but these techniques will also allow us to identify the degree of adulterations.

4.1 INTRODUCTION

Swertia chirata (*S. chirata*; genteianace*)* is one of the most important herb used in traditional medicinal system across India and is distributed throughout the temperate Himalayan regions [4, 45]. *S. chirata* have broad range of medicinal properties due to which humans have extensity exploited this herb from its natural habitat [25]. High demand of this herb has led to its habitat destruction and excessive exploitation and therefore it is now severely threatened by extinction from its natural habitat [132]. This plant possess wide range of phytochemicals such as alkaloids, saponins, flavonoids, glycosides, xanthones etc. which results in its wide range of pharmacological activities and is accountable for its extensive use in the traditional medicinal system across Indian subcontinent [15, 133]

Although, physically different species of *Swertia* show remarkable resemblance and are very difficult to differentiate, there are extreme variations in the phytochemical or secondary metabolites present in these species. This extensive difference in the phytochemical profile results in the difference in pharmacological potential and medicinal values of these species. Since *S. chirata* is thought to be pharmacologically superior from its other species, it has been exploited extensively from wild resources, reached to the verge of extinction. Due to scarcity and high demand in pharmaceutical industry it is often adulterated with other species. This may affect the quality, efficacy, safety and therapeutic potency of *S. chirata*. However, no comparative study of chemical profiles of the adulterant species *S. chirata* has been performed so for. So, in this investigation we have conducted a comparative metabolomics based profiling of *S. chirata* and its adulterants. Such a study could also ascertain whether is it possible (and to which extent) use adulterant plant species as really substitutes of *S. chirata* which is near to extinction, If chemical profiles of adulterants are somewhat similar. So, in this investigation we have conducted a comparative metabolomics-based profiling of *S. chirata* and its adulterants.

We prepared a metabolic profile of *S. chirata* and its adulterants by using advanced techniques like GC-MS, LC-MS identified the phytochemical variations in the metabolic profile of various species and given statistical treatment to the data generated to have closer insight of the metabolite variation.

4.2 MATERIALS AND METHODS

4.2.1 PLANT MATERIALS AND ITS IDENTIFICATION

Plant species were collected between the month of July and September from the tropical Himalaya regions of Himachal Pradesh and Uttarakhand. The species were identified and authenticated by Mr. Kisan Lal, florists of India and Prof. Narain Singh Chauhan, (Former Professor and Head of Department of Forest Products) Dr. Y.S. Parmar University HP India. The whole plants were shade dried, finely powdered by using an electric blender and stored in airtight plastic container at room temperature for further analysis.

4.2.2 PREPARATION OF EXTRACTS

Plant material was washed under running tap water to remove dirt and other debris. Plant material was dried under shade until constant temperature was achieved, coarsely grounded and subjected to hydroalcoholic extraction using Soxhlet apparatus. Solvent system was prepared in the ration 3:1 (methanol to water) and extraction was continued for 48 h or until plant material got exhausted at temperature below 70ºC. Extract was filtered while hot and was concentrated under reduced pressure. The resulted concentrate was subjected to lyophilization and power was stored at 4ºC until used further.

4.2.3 METABOLITE PROFILE OF *S. CHIRATA* AND ITS ADULTERANTS

Metabolite profile of the *S. chirata* and its adulterants was analyzed by using GC-MS technique by using Thermo GC-TRACE ultra 5.0, Thermo MS DSQ-II. Plant extract samples were dissolved in chloroform. The GC-MS was done using TRACE TR-5 GC, having 30 m length and 0.25m film thickness. Mobile phase was helium gas (carrier gas) and the flow rate was standardized to 1000µl/min. The oven temperature was varied between 40°C and 250°C with the change of 5°C every min. Samples of *S. chirata* and its adulterants were injected in the volume of 1l per injection. All the plant samples were run fully at a range of 50-650 m/z. Wiley Spectral library search program was used to compare the outcome.

4.2.4 STATISTICAL ANALYSIS

Using SIMCA-P+ software (version 12.0; Umetrics AB, Kinnelon, NJ, USA), the resulting data sets from both GC-MS and LC-MS / MS processes were subjected to multivariate statistical analysis. The data were Pareto-scaled and an unattended analysis of the main component (PCA) was performed to explore variations in the dataset. To evaluate the significance of difference, Principal component analysis was used to identify and to have closer insight of metabolite variation among the different species used in the study. Variable

importance plot (VIP-plot) was prepared to identify the potential biomarker in particular herbal sample. For a better understanding and better depiction, MeV software (version 4.9.0) was used for heat maps and hierarchical cluster analysis.

4.3 RESULTS

4.3.1 GC-MS-profiling

Two key stages are involved for metabolomics data analysis. The first stage is to obtain readable data set from mass spectrometry signals. The second stage is to carry out statistical analysis, which makes it possible to compare the similar information produced from different analytical platforms. Different peaks in the MS spectra were due to the fragmentation pattern of the molecules with large structure and these peaks were observed at different m/z ratio depending upon the size of the fragment. A representative GC of is shown in fig 4.1. The observed peaks at different m/z ratio are considered as the fingerprints of the parent molecule and the molecule can easily be identified from the data library. Phytochemical investigation of the hydroalcoholic extract of *S. chirata* demonstrated the presence of several metabolites, which included flavonoids, saponins, alkaloids, reducing sugar, tannins, glycosides, cardiac glycosides, polyphenols, steroids, carbohydrates, proteins, amino acids and terpenoids. Likewise, GC-MS analysis of the hydroalcoholic extract also confirmed the presence of many metabolites in the hydroalcoholic extract of *S. chirata*.

Also, the large data set obtained after GC-MS was screened manually and revealed the presence of marker compounds which can be used to discriminate the various species from each other used in this study. It is noteworthy that the uncommon compounds between *S. chirata* and its widely used adulterant *Andrographic paniculata* and *S. chordata* was 33 and 37 respectively (Fig 4.2).Potential marker compounds depending upon their presence and absence between pair of the species are listed in table 4.1. The results of this analysis indicate that Tetradecanal, Methyl 3-hydroxy-2-oxobutanoate, 1,2-benzenedicarboxylic acidare the most potential marker compounds to differentiate *S. chirata* from its adulterants (Table 4.2). However a separate detailed study is required to standardize workable protocol.

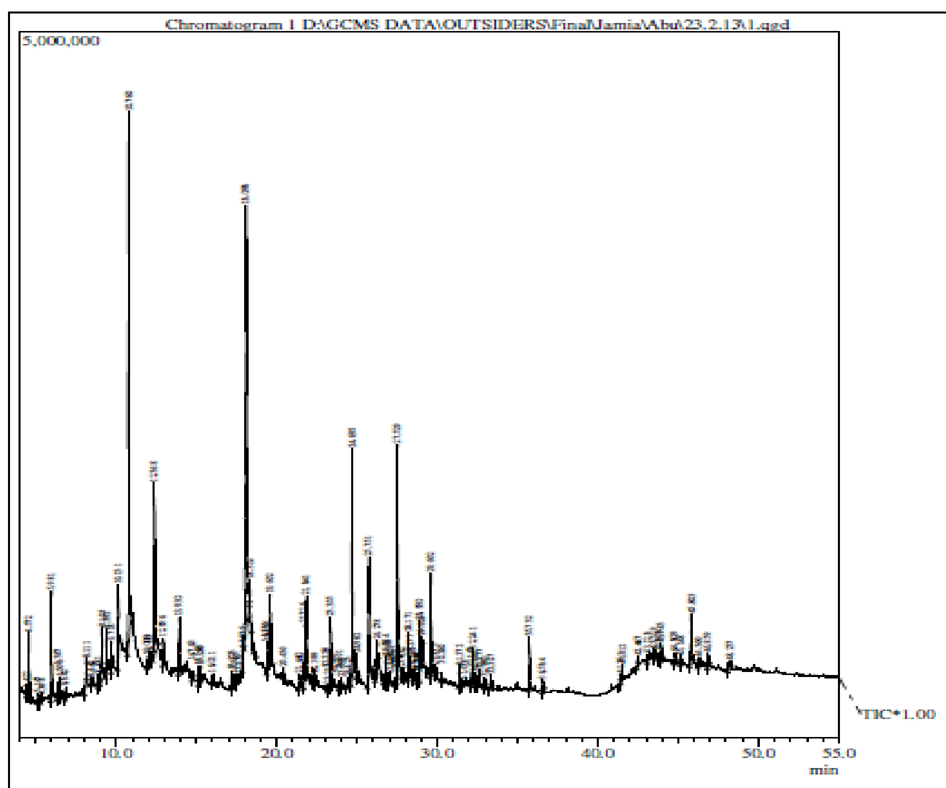


Figure 4.1: Standard GC-MS Chromatogram showing different peaks at different retention time

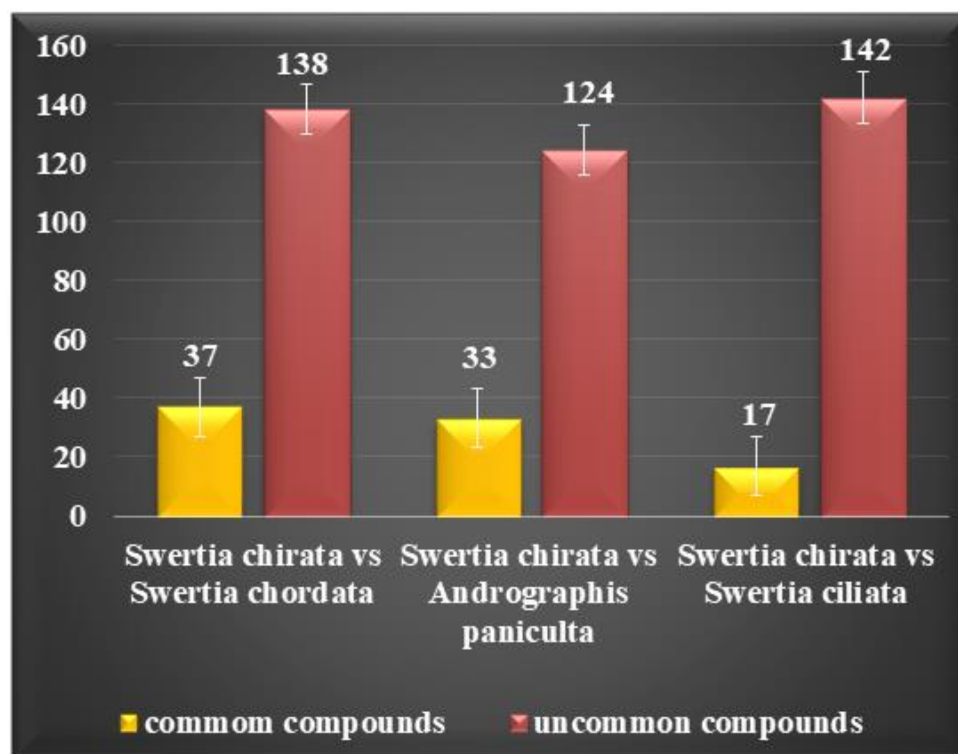


Figure4.2: Number of common and uncommon metabolites in different species with respect to *S. chirata*

Table 4.1: Potential Marker compounds which can be used to discriminate different pair of the *Swertia* species and adulterants

Species	Compounds name				
S. cordatavs A. paniculata	Tetradecanal	Methyl 3-hydroxy-2-oxobutanoate		Z-5-methyl-6-heneicosen-11-one	1-octadecanol
S. cordatavs S. chirata	Tetracontane-1,40-diol		Hexadecanoic acid, 2-hydroxy-, methyl ester		
S. cordatavs S. ciliata	Tetradecanal	Methyl 3-hydroxy-2-oxobutanoate	N-decanoic acid	4-dimethyl(prop-2-enyl) silyloxytridecane	
S. chiratavs A. paniculata	Tetradecanal	Methyl 3-hydroxy-2-oxobutanoate	1,2-benzenedicarboxylic acid	Z-5-methyl-6-heneicosen-11-one	1-octadecanol
A. paniculatavs S. ciliata	Tetradecanal	Methyl 3-hydroxy-2-oxobutanoate		Z-5-methyl-6-heneicosen-11-one	1-octadecanol
S. chiratavs S. ciliata	Tetradecanal	Methyl 3-hydroxy-2-oxobutanoate	N-decanoic acid	Z-5-methyl-6-heneicosen-11-one	

Table 4.2: Most Potential marker compounds which can be used to differentiate *S. chirata* from adulterant species

Species	*A. paniculata*	*S. cordata*	*S. ciliata*	*S. chirata*
Tetradecanal Methyl 3-hydroxy-2-oxobutanoate 1,2-benzenedicarboxylic acid				

4.3.2 STATISTICAL ANALYSIS OF GC-MS BASED METABOLOMIC DATA
4.3.2.3 PHYLOGENETIC ANALYSIS

The comparative evaluation of the metabolite profile of the three species revealed that *Swertia cordata* was found to have more common metabolites when compared to *A. paniculata* with respect to *S. chirata* (Fig 4.2). This was further confirmed by the dendrogram was obtained depicted in Figure 4.3 which revealed that *S. cordata* is more closely related to *S. chirata* in comparison to *A. paniculata.* Further dendrogram shows the evolutionary relationship between different *S. ciliata, S. chirata, S. cordata, A. paniculata* and mixture of all species. It was observed that the *S. chirata* was present in different clade whereas the samples of same species were clustered together. Also, the mixture sample was found closely related to *S. cordata* in comparison to *S. chirata*.

4.3.2.4 PRINCIPLE COMPONENT ANALYSIS (PCA) ANALYSIS

Although the discriminatory profile, phylogenetic tree indicate some of the potential markers, the statistical significance is not clearly shown. To avoid false identification of potential markers in metabolomics, the candidate markers must be strictly validated using appropriate statistical approaches [134]. So in this part of analysis GC-MS generated metabolomics data was subjected to assess by one-way analysis of variance. PCA was conducted to decrease the dimensionality of GC-MS generated metabolomics data. PCA is an unsupervised multivariate analysis method and this model provide us with a clear overview of the metabolomic data of all the analyzed species, which in our case happens to be *S. chirata* and its adulterants (Figure 4.4, Figure 4.5). In this model, samples with same metabolomic compositions were clustered together and are represented as a separate cluster, while those with different metabolic components are the one shown as dispersed. It was observed the three species which found that different components from each other as were dispersed on PCA plot.

4.3.2.5 VARIABLE IMPORTANCE PROJECTION (VIP) ANALYSIS

For each altered metabolite, variable importance projection value (VIP) was calculated and a cut-off point was made for all metabolites obtained from the GC-MS analysis. VIP was used to identify the potential biomarker in three species, *S. chirata*, *A.*

paniculata and *S. cordata*, which was based on a supervised OPLS-DA analysis technique (Figure 4.5). In these results, the value of VIP more than one depicts the importance of variables. Results are demonstrated in Figure 4.5b where potential metabolites are sorted in descending order based on their VIP values and their weighted sum of absolute regression coefficients. The metabolites with VIP values greater than one were deemed to be the most appropriate ones based on metabolite profiling for species discrimination.

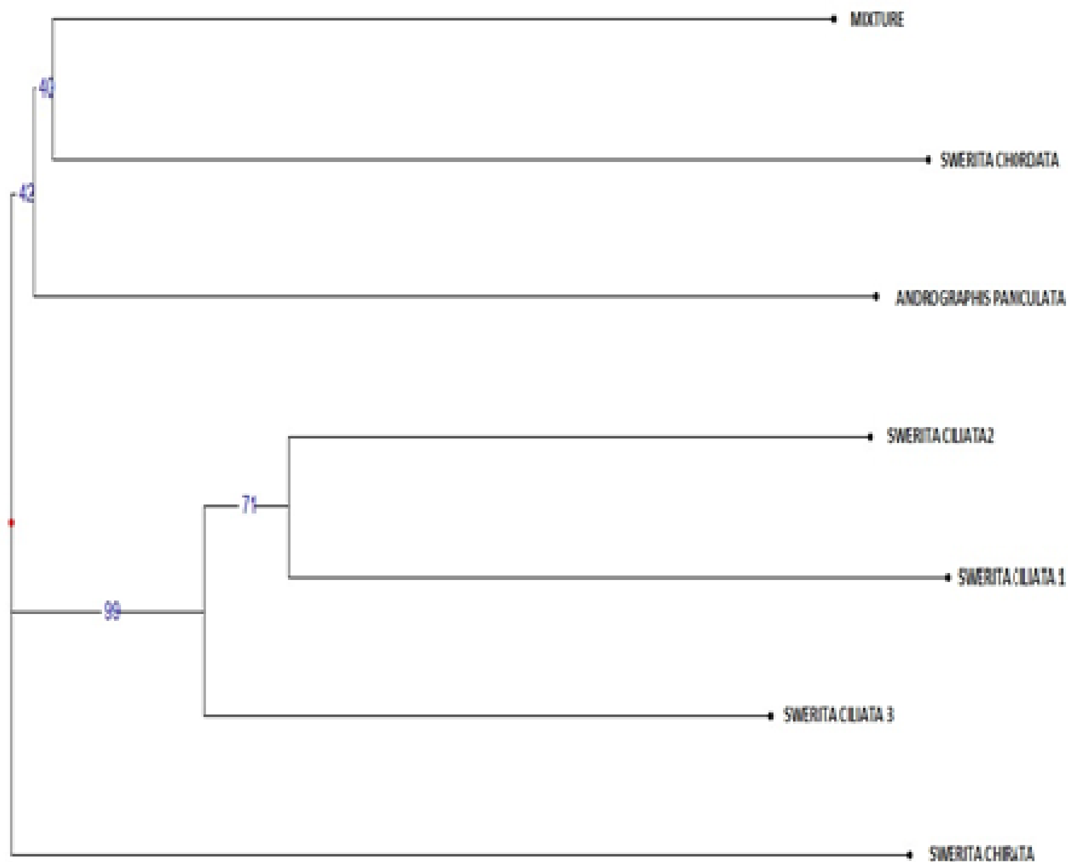


Figure 4.3: Dendrogram showing the evolutionary relationship between different species: *S. ciliata, S. chirata, S. chordata, A. paniculata* and Mixture of all species.

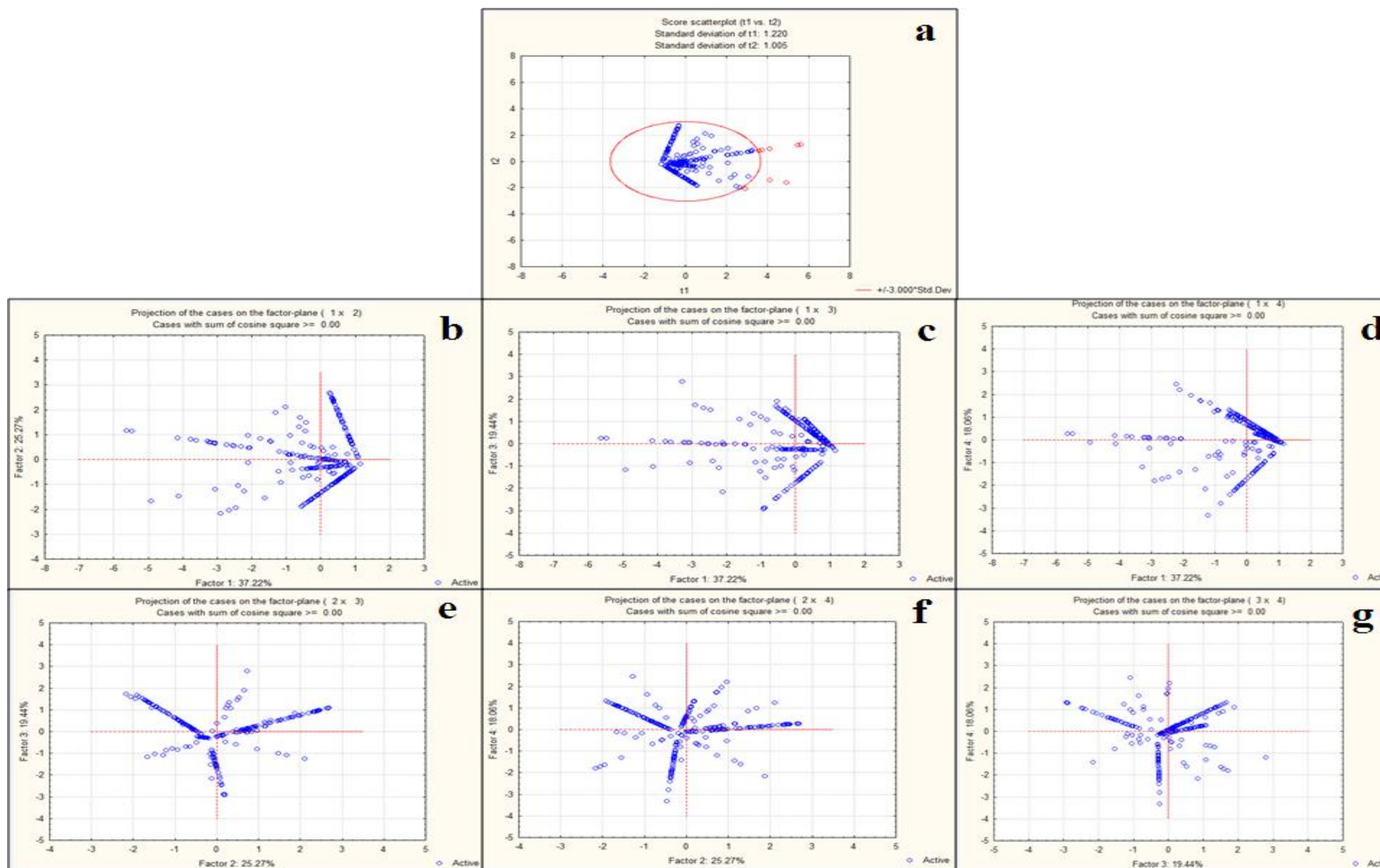


Figure 4.4:Comparative PCA analysis of the metabolites of four species i.e *S. Cordata,* 2- *S. chirata,* 3- *A. Paniculata,* 4- *S. Ciliata* as Scatter plots

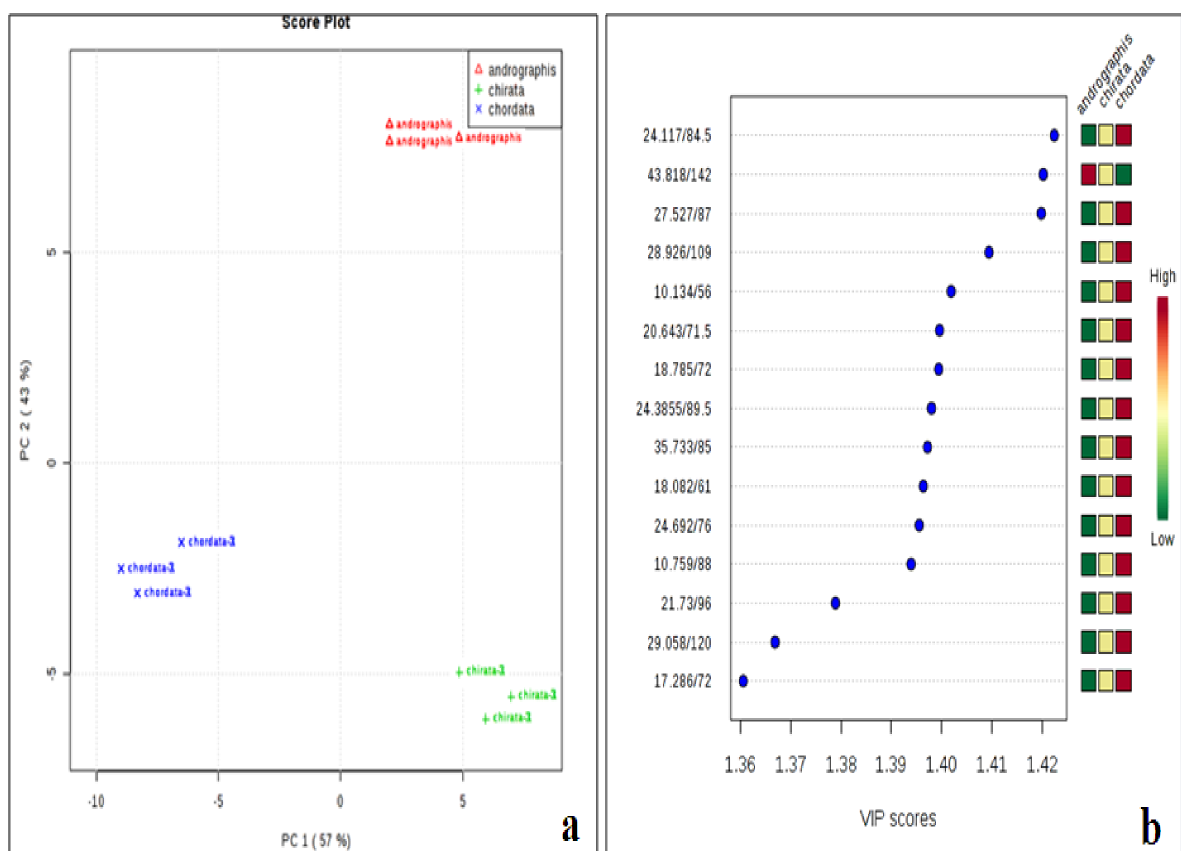


Figure 4.5: Principle component analysis (PCA) score plot (a) and variable importance in projections (VIP) scores (b) of 3 species of *Swertia*. The VIP plot is sorted in descending order. Coloured boxes: concentrations of the corresponding metabolite. Dash line: critical value.

4.4 DISCUSSION

The use of plants, plant preparations and phytochemicals has demonstrated remarkable health benefits in past and humans has been exploiting natural resources for the health benefits since ages. In recent years, the use of herbal preparations has demonstrated marked upgradation with the development of advanced techniques and technologies. In recent time, various methods have been used to prepare plant preparations for medicinal values, which includes, Soxhlet extraction, hot maceration, cold maceration, decoction, ultrasound-assisted extraction, supercritical fluid extraction and microwave-assisted extraction. Although, the efficiency of extraction and extraction technique depends on multiple factors such as pH, temperature, acidity, powder surface area, solvent to solute ratio, polarity, etc.,

these techniques has resulted in almost complete extraction of metabolites from plants and extraction of desired molecules as per the requirements [135]. Primarily, the appropriate selection of solvent system is crucial for the higher degree of extraction and the selection of solvent is dependent on the type of molecules or metabolites, which need to be extracted from the plant. For example, for the extraction of polar metabolites, the solvent system with higher polarity is recommended. However, for the complete extraction of plant metabolites, hydroalcoholic extraction using Soxhlet extractor is the most widely used technique [136], and we have used this technique to prepare the whole plant extract of *S. chirata* and its adulterants.

Further, the commonly used phytochemical laboratory test can only depict the presence of limited number of phytochemicals and they lack sensitivity and are only semi-qualitative [137, 138]. Therefore, to detect the present of phytochemicals or metabolites present in the extract in lower amounts, we need advanced and highly sensitive quantitative techniques. In this regard, GC-MS is an extremely reliable and useful technique. It has been utilized widely in the identification of plant metabolites present in various extracts. GC-MS offer advantage not only to identify the molecules which have been previously reported, but it also help us to identify unknown molecules based on their fragmentation pattern from where their structure can be elucidated [139]. A single run of GC-MS result in hundreds of peaks, and the analysis of compounds or molecules can be easily done by using the retention time and comparing it with the commercially available standards, if molecule of known [132, 136].

The biochemical pathways are highly regulated and complicated, which makes data interpretation of data mining a difficult task. Moreover, a significant amount of information is lost during averaging which also makes data interpretation difficult. Each plant possess an entirely different genotype and therefore and entirely different metabolites which correlates to its genotype. Therefore the understanding of metabolite gene correlation can be better understood by multivariate data mining techniques [140].

Following preprocessing and data normalization, the data is typically transformed into logarithm values to minimize the potential effects of outliers. To acquire biologically significant information, effective statistical discriminant analysis is then applied to the data set. Metabolite data was extracted using various pattern recognition techniques to classify data into groups, either knowing that classes exist, using supervised learning algorithms or

using unsupervised learning algorithms. Multivariate statistics deal simultaneously with the evaluation of various factors, including unsupervised methods of classification such as hierarchical cluster analysis (HCA), PCA, heat map, VIP Score and phylogenetic analysis.

In this regard, to compute an individual metabolic profile of each plant (*S. chitata* and its adulterants) we utilized the PCA to utilize all metabolite data from a plant samples. Moreover, PCS technique was also used to compare the observed metabolic profile of *S. chirata* with the metabolic profile of the plants which are used as its adulterants. Further, PCA pattern recognition was use for establishing genotype clustering, which confirmed the closely related species based on the observed clusters. This is an established and useful technique for demonstration gene function and associated metabolic profiles [141]. PCA utilize n-dimension vector approach and find basic vector, also known as eigen vector, which provide us with the best conditions for sample separation [142].Total variance is calculated, based on which vectors are determined by linear combination of all observed metabolite data. Therefore, PCA analysis assign plant samples with individual metabolic profiles to different groups, which are primarily defined by the genotype of individual plant. Therefore, through the use of PCA techniques different plants or adulterants can be identified.

In this study, we utilized and established GC-MS as an efficient and reliable approach to detect adulteration, not only with the plants of same genus but with the plants of other genus as well. GC-MS technique reveal several marker compounds that are specific to a particular plant or adulterant based on their genome, therefore help to identify adulterants in various preparation of the *S. chirata.* There are few limitations of this study. These limitations include the fact that the effect of temperature, precipitation and sunlight were not studied and these factors can result in the marked difference the quantity and the quality of the metabolites produced by plants.

4.5 CONCLUSION

The present study investigated the use of metabolic approaches to identify the metabolic variations between *S. chirata* and its adulterants. The indicate that Tetradecanal, Methyl 3-hydroxy-2-oxobutanoate, 1,2-benzenedicarboxylic acid are the most potential marker compounds to differentiate *S. chirata* from its adulterants. However a separate detailed study is required to standardize workable protocol. Phylogenetic analysis based on GC-MS generated metablomic data revealed that *S. cordata* is more closely related to *S. chirata* in comparison to *A. paniculata.* In PCA analysis samples with same metabolomic compositions were clustered together and are represented as a separate cluster, while those with different metabolic components were shown as dispersed. By utilizing these approaches, it is possible to efficiently identify not only the presence of adulterants in the herbal preparations of *S. chirata,* but these techniques will also allow us to identify the degree of adulterations. Further insight is required to devise a precise protocol for species discrimination on the basis of metabolite profiling.

CHAPTER 5

ANTIOXIDANT AND HEPATOPROTECTIVE EFFECT OF *SWERTIA CHIRATA* ON HYPOXIA-INDUCED OXIDATIVE STRESS IN WISTAR RATS

ABSTRACT

Swertia chirata have been used in traditional and folklore medicine to treat several ailments, such as hepatic disorders. However, the mechanistic and experimental justification to its traditional use is lacking. In our study the hepatoprotective potential of *S. chirata* during hypoxia-induced hepatic damage in Wistar rats was investigated and the underlying mechanism was determined. Hydroalcoholic extract of *S. chirata* was prepared using Soxhlet extraction. Animals were divided into 6 groups (n = 5). Animals in the hypoxia (HYP) groups were subjected to hypoxia for 3 days (10% O_2) to induce oxidative stress and hepatic damage. 50 and 100 mg/kg extract treatments were provided orally once daily for 7 days after which animals were sacrificed and biochemical investigations for oxidative stress, liver function tests, and hepatic histopathology was performed. HYP induced marked oxidative stress as indicated by the significantly elevated mitochondrial ROS generation, lipid peroxidation, glutathione and depleted catalase levels. Liver function test indicated hepatic damage as the amount of liver enzymes aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT), and ALP were significantly elevated in HYP animals. *S. chirata* treatment alleviated oxidative stress and improved liver functions in a dose-dependent manner. Liver histopathology confirmed the marked hepatic damage induced by hypoxia and revealed that *S. chirata* efficiently rescued liver from hypoxic damage. Hydroalcoholic extract of *S. chirata* is a potent hepatoprotective intervention which was associated with its ability to attenuate oxidative stress and improve liver functions. Moreover, it could find clinical application as a safer and alternative remedy for liver ailments.

5.1 INTRODUCTION

Oxidative stress has a central role in the occurrence and progression of a variety of ailments like hepatotoxicity, neurotoxicity, diabetes, Alzheimer's disorder, etc. [143, 144]. Our body is having an efficient antioxidant defense in from of catalase, glutathione, thiols, sulfoxide dismutase etc. which rapidly neutralize the reactive oxygen and nitrogen species [109]. However, our oxidative stress defense mechanism is a saturable process. When oxidative stress is increased in our body, especially during hypoxia and diabetes, ROS/RNS neutralizing capacity of antioxidant defense is saturated and ROS/RNS enter into the circulation. These free radicals interact with the biomolecules like DNA, RNA, proteins, lipids, etc. and inhibit their normal working leading to the growth of a various health ailments [109]. The liver is responsible for the metabolism and detoxification of a variety of drugs and toxins and therefore is on the higher risk in occurrence of oxidative stress-mediated toxicity [145]. Oxidative stress is related with the occurrence of liver toxicity and liver illness such as alcoholic liver disease and non-alcoholic steatohepatitis [110]. Moreover, oxidative stress is markedly enhanced during hypoxic state which further deteriorates the liver functions and is associated with the development and progression of liver complications [146-148].

To date, there is no drug available which can counter oxidative stress and associated complications. There is an urgent need for the screening of some safer and alternative therapeutic strategies which can effectively counter oxidative stress and therefore may attenuate associated complications. Plants have long been exploited as a source of safe and efficient therapeutic agents and may provide us with the needed antioxidant therapy which can not only alleviate the oxidative stress but may also halt the progression of ROS and RNS mediated complications [149]. *S. chirata* has long been used in Ayurvedic and Unani medicine as an anti-helminthic, febrifuge, stomach and liver tonic [119, 150]. This plant is abundant with a large number of alkaloids and flavonoids along with other different phyto-constituents that are responsible for its all broad range of medicinal properties [151]. Research work focused on *S. chirata* suggests that plant extract is beneficial in the management of liver disorders, however, the mechanism through which hepatoprotection is achieved remains unknown to a largeextent. In this study, we aimed to provide scientific evidence to the traditional use of *S. chirata* and investigated whether or not plant extract is

beneficial in the management of hypoxia-induced hepatotoxicity and investigated its effect on oxidative stress as a potential mechanism.

5.2 MATERIAL AND METHODS

5.2.1 MATERIAL

In this study all the chemicals and reagents used were of analytical grade procured from Sigma, Hi Media, and Loba Chemie., unless otherwise specified. Autoanalyzer P800 Hitachi Modular was used for evaluation for serum biochemical parameters. Kits for biochemical analysis were used from Roche Diagnostics Roche Diagnostics, Japan. 5, 50-dithiobisnitrobenzoic acid (DTNB) and 2-thiobarbituric acid (TBA) were procured from the Sigma Chemical Co., USA.

5.2.2 SAMPLE COLLECTION AND EXTRACTION

Plant species were collected from high altitude regions of Himachal Pradesh and Uttarakhand from the month of July–September. Identification and authentication of the plants was done by Prof. (Dr.) Narayan S. Chauhan, Dr. YSP University, Solan, Himachal Pradesh, India. For the preparation of the extract whole plant was used. The samples were washed, shade dried at room temperature and coarsely powdered. Extraction was performed in a Soxhlet apparatus at 25°C for 72 h in methanol: water 20:80. The hot extract was filtered and finally lyophilized after concentrated under reduced pressure by rotary evaporator. Extract yield was measured and the extract was preserved at -20°C in a refrigerator until used for further analysis.

5.2.3 ANIMALS

All experimental protocols were carried out on male Wistar rats strictly following the guidelines after permission from the Institutional Animal Ethics Committee (DIHAR/IAEC/36/2015). Wistar rats (150-200g; 8-10 month age) were housed in the DIHAR animal house inside polypropylene cages (2 animals per cage) at the temperature of 23±2ºC, 12h day/light cycle and 65±5% relative humidity. All desired precautions were taken to lower down the sufferings to the animals. The dose selection was based entirely on the literature. This was made after considering the ethics in animal use and the suggestions of the Institute Animal Ethics Committee as per the guidelines of the CPCSEA, Government of India.

5.2.4 ANIMAL GROUPINGS

Animals were grouped into 6 groups having 5 animals in each group as follows:

Group 1- Control (received 0.3% carboxymethyl cellulose as a vehicle orally)

Group 2- Control + 50mg/kg *S. chirata* extract suspended in a vehicle (Control 50)

Group 3- Control + 100 mg/kg *S. chirata* extract suspended in a vehicle (Control100)

Group 4- Hypoxia (HYP) (rats exposed to 10% oxygen for 3 days and received vehicle orally)

Group 5-HYP+50mg/kg *S. chirata* extract suspended in a vehicle (HYP 50)

Group 6-HYP+ 100 mg/kg *S. chirata* extract suspended in a vehicle (HYP 100)

Mode of treatment was oral given once a day between 6-7 pm for 7 days.

5.2.5 INDUCTION OF HYPOXIA

Hypoxic conditions were provided to the animals by placing them inside a custom-made hypoxia chamber linked with oxygen (O_2) and nitrogen (N_2) cylinders. The concentration of O_2 was regulated by infusing N_2 into the chamber. The hypoxic treatment was given to the rats in the hypoxia chamber at 10% O_2 for 3 days.

5.2.6 ANIMAL SACRIFICE AND SAMPLE COLLECTION

Animals were sacrificed by cervical dislocation and tissues were collected for histopathological examination and biochemical analysis. For biochemical evaluation, tissue samples were weighed, homogenized in HEPES buffers and stored at 4°C until used. Blood was collected and serum was isolated for the evaluation of liver function tests (enzymes). All the samples were used within 6 h for the biochemical evaluations. For histopathological examinations, tissues were fixed by double circulation by infusing phosphate buffer saline (PBS; pH 7.4) followed by a fixing solution of 2% glutaraldehyde and 2 % formaldehyde. Tissues were collected and stored in fixing solution at room temperature.

5.2.7 BIOCHEMICAL EVALUATION

5.2.7.1 MITOCHONDRIA ISOLATION

Mitochondria were isolated using a method described by Brown et. al. (2004) [153], with slight modifications. Briefly, dissected tissue was weighed and homogenized by Teflon hand homogenizer in homogenizing buffer (five volumes). Homogenizing buffer consisted of 1 mM ethylene glycol tetra acetic acid (EGTA), 215 mM mannitol, 75 mM sucrose, 0.1 %

bovine serum albumin (BSA), 20 mM hydroxyethyl piperazine ethane-sulfonic acid (HEPES) and pH adjusted to 7.2 with potassium hydroxide. Homogenate was centrifuged at 10,000 g at 4ºC for 5 min. The resulting pellet was suspended in 500µl homogenizing buffer. In order to pellet out the mitochondria, centrifugation was carried out again at 13,000g for 10 min. The pellet obtained after centrifugation was washed in the EGTA containing buffer and centrifuged at 10,000g for 10 min, and suspended in the same buffer at a concentration of 10 mg/ml.

5.2.7.2 MITOCHONDRIAL ROS GENERATION

Mitochondrial ROS generation was evaluated in accordance with the method described by [154], with some necessary modifications. Briefly, approximately 10 µl of mitochondria (15 µg protein equivalent) were incubated with the respiration buffer containing 5 mM pyruvate, 2.5 mM malate, and 10M of dichloro-dihydro-fluorescein diacetate (H_2DCFDA) for 20 min at 37ºC. After incubation, fluorescence as a result of ROS generation was quantified using a Cary Eclipse fluorimeter (Varian, Palo Alto, USA) (excitation 485nm, emission 582 nm) and related to total protein content. Thereafter carbonylcyanide-4-(trifluoro methoxy)-phenyl hydrazone (FCCP) was added to inhibit the interference from the membrane potential-dependent ROS production.

5.2.7.3 LIPID PEROXIDATION

Lipid peroxidation was assayed by the method of Mehta et. al. (2017) [155], with some necessary modifications. Briefly, 500 µl of mitochondrial extract equivalent of 15 µg protein was diluted with equal volume of Tris-HCl buffer (0.1 M, pH 7.4). Incubation of reaction mixture for 2 h at 37°C with constant shaking inside an incubator. Trichloro acetic acid (1000ul 10%w/v) was added to the reaction mixture, mixed thoroughly and subjected to centrifugation at 8000 rpm for 10 min. 1500µl 0.67 % w/v thiobarbituric acid was added to each tube and tubes were heated to 100°C for 10 min in a boiling water bath. Reaction mixture were cooled under tap water (RT), followed by centrifugation at 10000 rpm for 15 min and the absorbance of the supernatant was recorded spectrophotometrically at 532 nm.

5.2.7.4 CATALASE ACTIVITY

Catalase activity was assayed spectrophotometrically at 240 nm and the results were expressed as moles of hydrogen peroxide (H_2O_2) consumed as per the method described by Mehta et. al. (2017) [155]. The reaction mixture consisted of a 500µl brain homogenate equivalent of 15 µg protein. The reaction was initiated by the addition of 32 µl of 30% H_2O_2.

Absorbance was recorded every minute for 4 minutes using UV spectrophotometer and the level of catalase in the homogenate was determined in terms of the amount of H_2O_2 consumed during the 4-minute reaction interval.

5.2.7.5 GLUTATHIONE PEROXIDASE ASSAY

Glutathione peroxidase activity was determined with some modifications as per the requirements. The reaction mixture consisted of 400µl of 0.35 M phosphate buffer (pH 7.4), 100µl of 10 mM sodium azide, 200µl of 8 mM freshly prepared glutathione (GSH), 500µl mitochondrial sample equivalent to 15 µg protein, 100µl 2.5mM H_2O_2, and 1100µl double distilled water. The reaction mixture was incubated for 5 min at 37ºC. 500µl of 10% trichloroacetic acid was added to the reaction mixture followed by centrifugation at 3500 rpm for 15 min at room temperature. 500µl supernatant was diluted with 3000µl of 0.3 M disodium hydrogen phosphate. 1000µl 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) (freshly prepared in phosphate buffer pH 7.4) was added to the reaction mixture. The reaction mixture was allowed to stand for 5 min at room temperature and then its absorbance was recorded at 412 nm using a UV spectrophotometer. The results were expressed as mg of GSH consumed/min/mg protein.

5.2.8 LIVER FUNCTION TEST

Serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and serum aspartate transaminase (ALP) are the extensively studied biomarkers of the hepatic damage in both, clinical as well as pre-clinical settings. Liver function tests were performed to evaluate the effect of *S. chirata* extract in hypoxia-induced liver damage in term of SGOT, SGPT and ALP levels. Levels of these enzymes were measured by using commercially available kits as per the manufacturer's instructions.

5.2.9 HISTOPATHOLOGICAL EXAMINATION

Histopathological examination of the liver tissue was performed to evaluate the hypoxia-mediated liver damage and to evaluate the hepatoprotective potential of hydroalcoholic leaf extract of *S. chirata* as per the method described by Aboonabi et. al. (2014) [155]. Briefly, animals were anesthetized and tissue was fixed by double circulation technique by infusing phosphate buffer saline (pH 7.4) followed by a fixing solution of 2% glutaraldehyde and 2 % formaldehyde. Tissues were collected and stored in fixing solution at room temperature. Tissues were embedded into the paraffin wax and 5 µm thick section were prepared by using microtome. Sections were stored at room temperature and

histopathological alterations were detected by staining sections with hematoxylin and counterstaining with eosin (hematoxylin-eosin staining). Effect of hypoxia and extract treatment on cellular morphology of liver was assessed by analyzing images captured at 100X magnification.

5.2.10 STATISTICAL ANALYSIS

All the statistical analysis were carried out by Graph Pad Prism 6. All values are expressed as mean ± SEM. Statistical significance was calculated by one way ANOVA followed by Dunnet post hoc test at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs control and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs HYP.

5.3 RESULTS

5.3.1 EXTRACTIVE YIELD AND PHYTOCHEMICAL EVALUATION

The extractive yield of the hydroalcoholic extraction of *S. chirata* was observed to be 22.37% of the crude plant sample. Phytochemical screening was performed and it revealed the presence of flavonoids, alkaloids, glycosides, steroids, terpenoids, tannins and saponins. Volatile oil, fixed oil, proteins and acidic compounds were not present in the plant extract. Moreover, we observed the high amount of phenolic and flavonoids present in the plant extract which might be responsible for its antioxidant potential.

5.3.2 BIOCHEMICAL INVESTIGATION

5.3.2.1 MITOCHONDRIAL ROS GENERATION

Mitochondrial ROS generation and the effect of hydroalcoholic extract of *S. chirata* on it were determined and the results are presented in Fig. 5.1 (A). Animals exposed to hypoxic stress produced high levels of oxidative stress as suggested by the significantly ($p < 0.001$) high levels of ROS generated in the HYPgroup in comparison to CTRL. ROS levels differed non-significantly in control animals treated with plant extract at 50 mg/kg and 100 mg/kg dose, when compared to CTRL, suggesting that plant extract is not associated with enhancing ROS production directly. Further, treating hypoxic rats with 50 mg/kg and 100 mg/kg dose of *S. chirata* extract significantly ($p < 0.01$) lowered the generation of mitochondrial ROS in a dose-dependentmanner when compared to hypoxia animals. These results suggest that *S. chirata* extract is having good antioxidant potential which may aid it the beneficial effects associated with extract treatment in the animals.

5.3.2.2 LIPID PEROXIDATION

Malondialdehyde (MDA) levels are used to determine the levels of lipid peroxidation in the biological samples, especially in the animal experimentation. This method was used to evaluate the effect of *S. chirata* extract treatment on the lipid peroxidation in hypoxic rats and the results are depicted in Fig. 5.1 (B). We observe a significantly ($p < 0.001$) high levels of MDA or lipid peroxidation in HYP animals in comparison to CTRL, suggesting the occurrence of oxidative stress in HYP animals. Treating HYP animals with plant extract resulted in a dose-dependent lowering of the lipid peroxidation levels at 50 mg/kg ($p < 0.05$) and 100 mg/kg ($p < 0.01$) dose. We did not observe any significant lipid peroxidation in control animal treated with plant extract and the MDA levels in these groups were similar to CTRL, suggesting that plant extract is not associated with lipid peroxidation directly.

5.3.2.3 CATALASE ACTIVITY

Catalase is a strong antioxidant enzyme present in our body which is responsible for the detoxification of the ROS generated from the electron transport chain in the mitochondria. We evaluated the effect of *S. chirata* extract treatment on the catalase activity during hypoxia in Wistar rats and the results are presented in Fig.5.1 (C). Our findings revealed that hypoxia leads to the saturation of catalase enzyme and its levels were observed to be significantly ($p < 0.001$) lower in HYP animals in comparison to CTRL. Hypoxic animals treated with the plant extract resulted in a dose-dependent improvement in catalase levels and catalase activity was observed significantly higher in HYP animals treated with 50 mg/kg ($p < 0.05$) and 100 mg/kg ($p < 0.01$) plant extract when compared to CTRL. Catalase levels were reported normal in control animals treated with extract in comparison to CTRL. These results indicates that *S. chirata* possess an antioxidant potential which can be partially attributed to its potential to either upregulate catalase activity or prevent its depletion during oxidative stress.

5.3.2.4 GLUTATHIONE PEROXIDASE ASSAY

To estimate the level of oxidative stress in the body determination of the glutathione levels is an established method. The effect of *S. chirata* extract treatment on the glutathione during hypoxia in Wistar rats was evaluated and the results are presented in Fig.5.1 (D). Our finding revealed that hypoxia leads to marked oxidative stress as depicted by the remarkably ($p < 0.01$) decreased glutathione levels in HYP animals in comparison to control. Hypoxic animals treated with the plant extract give rise to a dose-dependent elevation in glutathione levels at 50 mg/kg ($p < 0.05$) and 100 mg/kg ($p < 0.05$), when compared to HYP. Normal animals treated with extract showed glutathione levels comparable to control.

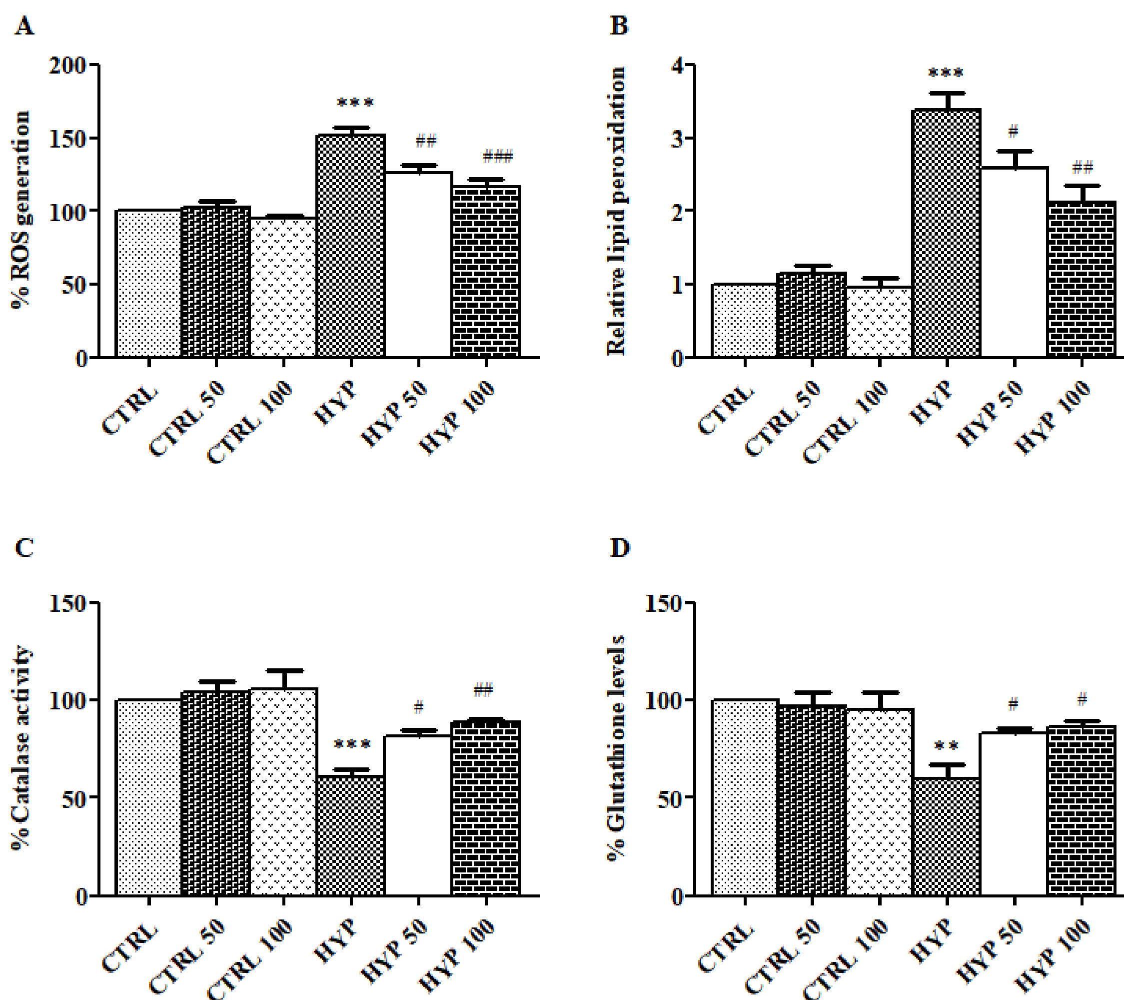


Figure 5.1: Effect of *S. chirata* treatment on hypoxia induced oxidative stress (A- % ROS generation; B- lipid peroxidation; C- % catalase activity; D- % glutathione levels). Values are represented mean ± SEM. CTRL- control; CTRL 50- control + 50 mg/kg extract; CTRL 100- control + 100 mg/kg extract; HYP- hypoxia; HYP 50- hypoxia + 50 mg/kg extract; HYP 100- hypoxia + 100 mg/kg extract. *p < 0.05; **p < 0.01; ***p < 0.001 versus CTRL group. $^{\#}$p < 0.05; $^{\#\#}$p < 0.01; $^{\#\#\#}$p < 0.001 versus HYP group.

5.3.3 LIVER FUNCTION TESTS

Hypoxia-induced hepatic damage and the hepatoprotective effect of *S. chirata* treatment was determined by evaluating liver function tests in which we determined the amount of biochemical liver enzymes such as SGOT, SGPT, and ALP(Fig. 5.2). Significant increase ($p < 0.001$) in the serum levels of SGOT, SGPT, and ALP, indicated the marked damage of hepatic cells in case of hypoxia. Extract treatment exerted a dose-dependent effect on hepatic biochemical enzymes. Levels of SGOT, SGPT and ALP were reduced significantly by the extract treatment at 50 mg/kg ($p < 0.05$) and 100 mg/kg ($p < 0.01$) extract treatment. These results suggest that extract treatment efficiently protected hepatic cells from

hypoxia-mediated damage. Moreover, extract treatment had no hepatotoxic effect of its own as the levels of SGOT, SGPT and ALP were observed to be non-significantly different from control animals.

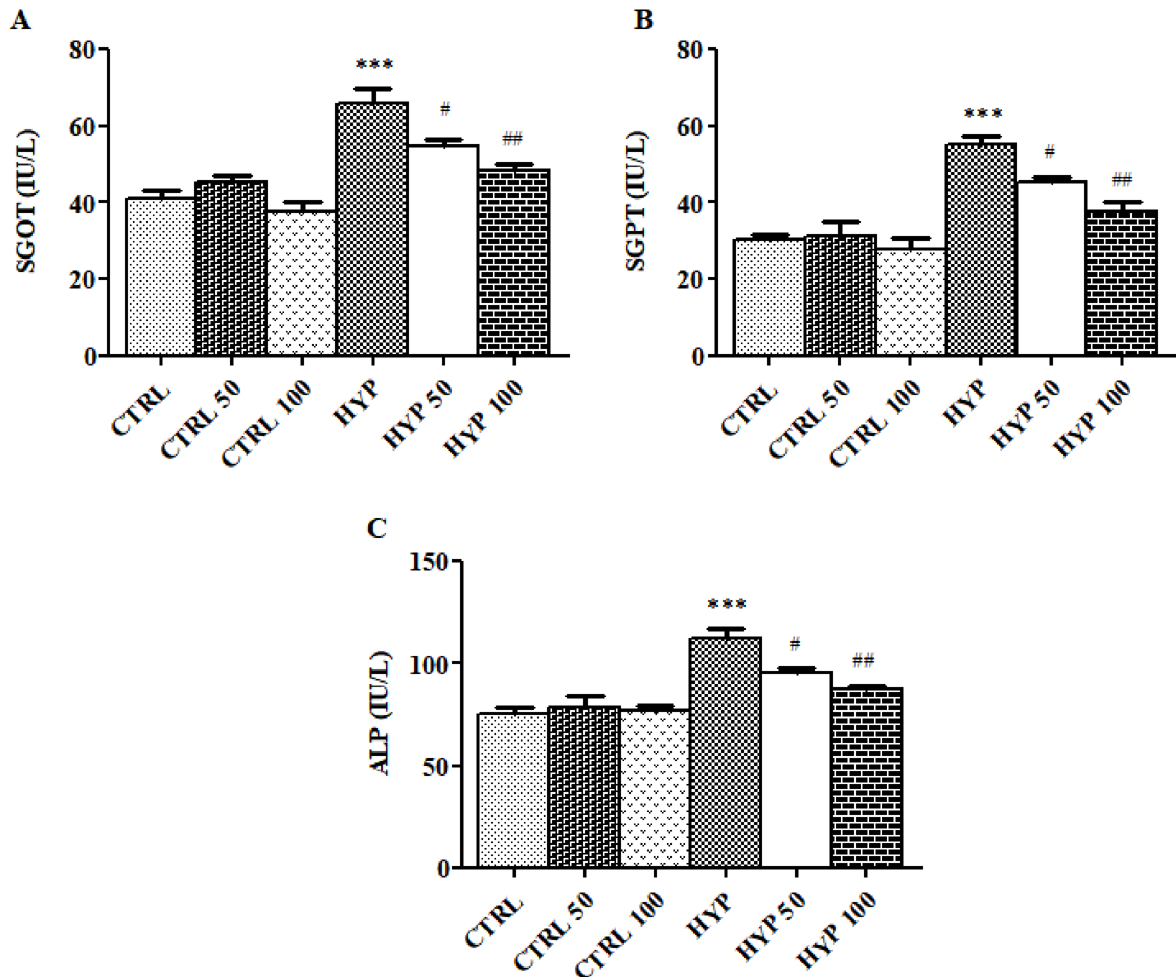


Figure 5.2: Effect of *S. chirata* treatment on hypoxia induced hepatic damage (Liver Function Test). A- SGOT (IU/L); B- SGPT (IU/L); C- ALP (IU/L). Values are represented mean ± SEM. CTRL- control; CTRL 50- control + 50 mg/kg extract; CTRL 100- control + 100 mg/kg extract; HYP- hypoxia; HYP 50- hypoxia + 50 mg/kg extract; HYP 100- hypoxia + 100 mg/kg extract. *p < 0.05; **p < 0.01; ***p < 0.001 versus CTRL group. $^{\#}$p < 0.05; $^{\#\#}$p < 0.01; $^{\#\#\#}$p <0.001 versus HYP group.

5.3.4 HISTOPATHOLOGICAL EXAMINATION

We further confirmed the hypoxia-mediated liver damage and the effect of extract treatment on it through histopathological examination of the Liver using hematoxylin-eosin staining. We observed a normal architecture of hepatic cells in control animals, cells appeared to be healthy and had a normal physiological appearance with no signs of damage

or cytotoxicity. We further observed a marked liver damage in animals subjected to hypoxia (HYP). Cellular morphology was distorted and signs of significant cell death, cellular damage and necrosis were observed in these animals. Treating hypoxic animals with plant extract resulted in significant hepatoprotective effect, which was comparable at both the treatment dose. Hepatic cells appeared healthy with significantly lesser signs of cellular damage, cell death, andnecrosis when compared to HYP. Extract treatment efficiently protected hypoxia-mediated cellular damage in the liver. Moreover, *S. chirata* was not having any hepatotoxic effect of its own at the given doses as indicated by the normal cellular morphology and histopathological appearance of control animals treated with the plant extract which was comparable to control.

5.4 DISCUSSION

The present investigation demonstrated the antioxidant and hepatoprotective potential of hydroalcoholic extract of *S. chirata* against hypoxia-induced oxidative stress in Wistar rats. During hypoxia, excessive amount of ROS are generated by the mitochondria which gives rise to the development of oxidative stress. Once Reactive oxygen species generation go beyond the normal limit, the neutralizing capacity of antioxidants present in our body, these highly reactive free radicals react with biomolecules such as lipid, protein, DNA etc. which lead to cellular dysfunction [109, 110]. ROS disrupts the functions of the cellular membrane by interacting with membrane phospholipids and inducing lipid peroxidation [109, 110, 155]. Our biological system is having natural antioxidants in form of catalase and glutathione, which efficiently neutralize the ROS and protects biomolecules from ROS mediated damage. However, these antioxidants are saturable and during excessive oxidative stress, as observed during hypoxia, fails to impart complete protection and adverse effects of

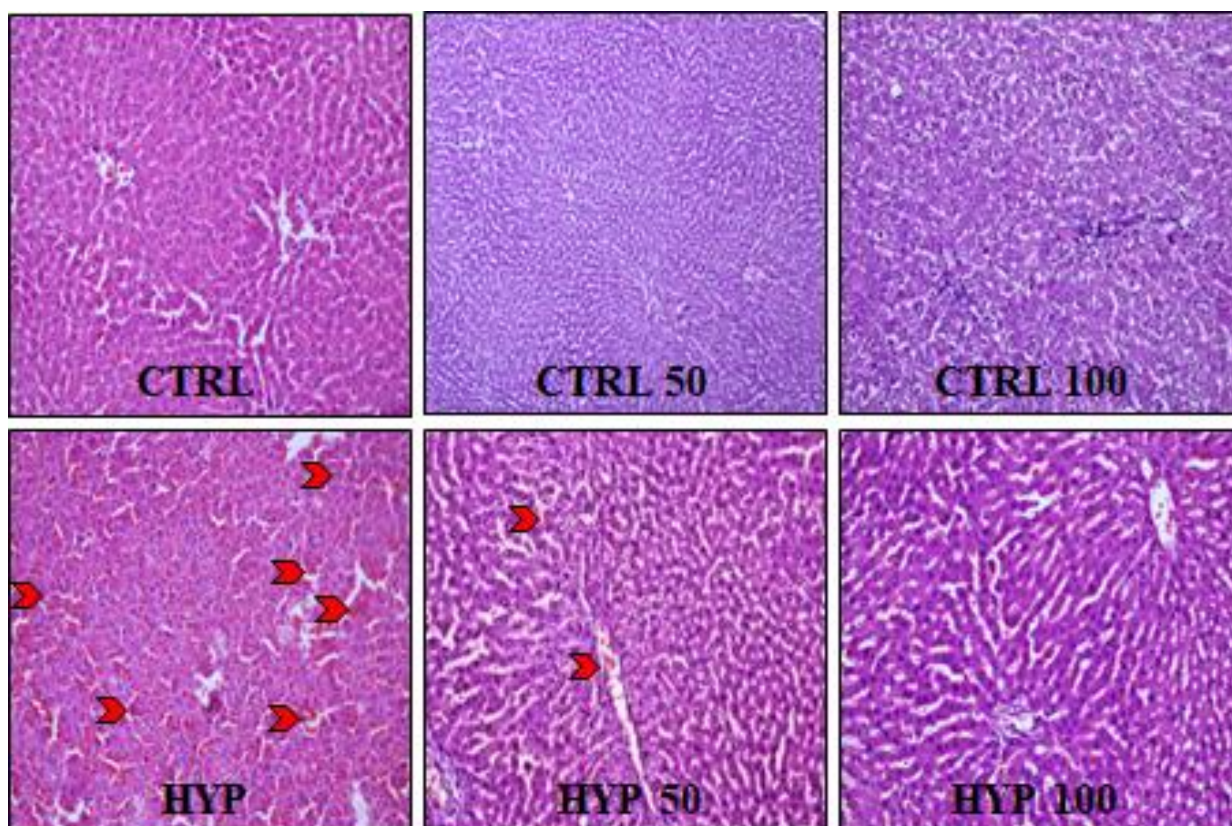


Figure 5.3: Effect of *S. chirata* treatment on hypoxia induced liver damage. Histopathological examination of liver using hematoxylin-eosin staining. Red arrow indicate the damage regions on the histopathological sections. CTRL- control; CTRL 50- control + 50 mg/kg extract; CTRL 100- control + 100 mg/kg extract; HYP- hypoxia; HYP 50- hypoxia + 50mg/kg extract; HYP 100- hypoxia + 100 mg/kg extract.

oxidative stress are inflicted [109,110, 152-156]. Oxidative stress is related with the occurrence of hepatotoxicity [110], cellular damage, necrosis [157], apoptosis [158], and disorders like dementia, Alzheimer's, etc. [159]. Previous reports suggest that the hypoxic state is related with the development of hepatic toxicity and impaired hepatic functions [110, 160]. Our results are in agreement with these reports and we have demonstrated that hypoxia is related with enhanced oxidative stress and hepatotoxicity. Therapeutic interventions of natural origin which have ability of attenuating oxidative stress are studied to exert hepatoprotective and other beneficial effects [161, 162, 163]. Our findings are in line with these reports and we demonstrated that hydroalcoholic extract of *S. chirata* possess strong antioxidant potential as indicated by the reduced ROS generation, improved catalase activity, enhanced glutathione levels and inhibition of lipid peroxidation (Fig. 5.1), which was associated with the improved hepatic functions.

Plants and herbal preparations are well-knownsources of bioactive antioxidants, such as flavonoids, which have been reported to be beneficial during various indications such as hepatotoxicity [161-163]. Phytochemical screening of the hydroalcoholic extract of *S. chirata* revealed the presence of a high amount of flavonoids, which may be responsible for its antioxidant and hepatoprotective potential. *S. chirata* is an important medicinal plant and it has been used traditionally for the management of various ailments such as hepatotoxicity [164]. Likewise, various herbal preparations are well documented for their hepatoprotective potential and are known to exert their beneficial effects by improving the morphology and biochemical profile of liver [165-167]. SGOT, SGPT and ALP are the biomarkers of liver damage and are elevated during liver toxicity [168]. Hypoxia is known to elevate these enzymes and induce morphological changes in the liver [169]. Various herbal preparations impart their hepatoprotective effects by reducing the levels of these enzymes [165, 170, 171] and improving cellular morphology [165-167]. Our results are in accordance with these reports. We have exhibited that hypoxia-induced marked cellular damage in the liver which was associated with cellular damage and significantly elevated hepatic enzymes (Fig. 5.2 and Fig. 5.3). Moreover, like previously reported herbal preparations, *S. chirata* efficiently improved liver morphology and improved the biochemical profile of the liver. These results suggest that hydroalcoholic extract of *S. chirata* is having marked hepatoprotective potential which attributes to its ability to attenuate hypoxia-induced oxidative stress in the biological system.

5.5 CONCLUSION

Hypoxia is associated with the development of hepatic damage, enhanced oxidative stress, altered hepatic morphology and disrupted hepatic biochemical profile in Wistar rats. The hydroalcoholic extract of *S. chirata* demonstrated marked hepatoprotective potential which was associated with the attenuation of hypoxia-mediated oxidative stress. Our finding provides an experimental justification for the traditional use of this plant during the liver disorders. Moreover, herbal preparations are safe to use and may find a clinical application for the management of hepatic ailments associated with oxidative stress. These findings are preliminary and need to be evaluated further.

CHAPTER 6

HYDROALCOHOLIC EXTRACT OF *SWERTIA CHIRATA* AND *SWERTIA CORDATA* ATTENUATE HYPOXIA MEDIATED MEMORY DYSFUNCTION BY IMPROVING NEURONAL SURVIVAL IN WISTAR RATS

ABSTRACT

Swertia chirata and *S. cordata* have been used in traditional and folklore medicine to treat several mental disorders. However, the mechanistic and experimental justification to its traditional use is lacking. The current study was designed with the aim to investigate the neuromodulatory potential of *S. chirata* and *S. cordata* during hypoxia-induced neuronal damage in Wistar rats and to determine the underlying mechanism. Animals were divided into 6 groups (n = 5). Hypoxia was inflicted by subjecting animals to the atmosphere having 10% O_2 for 3 days. Animals were administered 100 mg/kg hydroalcoholic extract of *S. chirata* and *S. cordata* orally once daily for 7 days after which motor coordination by rota-rod test and memory functions by active and passive avoidance tests were evaluated. Animals were sacrificed and biochemical investigations for oxidative stress and histopathology were performed. Hypoxia treatment resulted in marked increase in oxidative stress as indicated by the significantly elevated reactive oxygen species and lipid peroxide levels. *S. chirata* and *S. cordata* pretreatment prevented hypoxia induced oxidative stress generation in the hippocampus region of the rat brain. Further brain histopathology was studied to observe if hypoxia exposure resulted in any morphological differences in neuronal cells and it was found to result in significant neuronal damage as evidenced by haematoxylin and eosin staining. Pretreatment with plant extracts efficiently rescued rat neuronal cells from hypoxic damage. In comparison between the two plant extracts, *S. chirata* extract pretreatment was observed to have better neuromodulatory effect than *S. cordata.* Thus from this study we inferred that hydroalcoholic extract of *S. chirata* and *S. cordata* prevented memory impairment hypoxia treated rats counteracting oxidative stress production by increasing antioxidant enzymes levels (catalase and glutathione) and decreasing lipid peroxides levels, which thereby prevent neurodegeneration as observed by haematoxylin and eosin staining.

6.1 INTRODUCTION

Hypoxia represents deprivation of adequate amount of oxygen to tissues and organs of body which is caused due to a reduced partial pressure of oxygen in inspired air. Intermittent hypoxia is generally accepted as "repetitive hypoxia interspersed with episodes of normoxia" [172]. Oxygen homeostasis is essential for the development and physiology of an organism which is regulated by hypoxia inducible factor-1 (HIF-1) [173]. HIF-1 protein has a half-life of 5 min and is normally degraded through the ubiquitin–proteasome pathway but is quite stable under hypoxic conditions [174]. Apart from playing role in ischemia and vascular development, hypoxia signal transduction pathway plays a major part in neurodegeneration [175]. Hypoxia environment particularly affects neurons in the hippocampus and neocortex [176]. During normal aging, HIF-1 is up-regulated in the human frontal cortex [177]. Environmental or physiological changes leads to variation in cellular homeostasis which has a severe impact on mammalian brain. Oxygen transport to cells and tissues is impaired with the advancement of age, thereby increasing the susceptibility of neurons to damage. The parts of brain, actively involved in cognition requires constant supply of energy, hence oxygen. Any hypoxic exposure to brain results in pathological changes such as those seen in Alzheimer Disease [155, 178]. Due to hypoxia, mitochondrial electron transport chain generates excessive free radicals, such as peroxynitrite, superoxide, and hydroxyl radicals [179]. These free radicals are neutralized by the antioxidants and radical scavenging enzymes present in body; however, neutralizing capacity of these mechanisms is limited and therefore gets saturated leading to the development of oxidative stress. These excessive free radicals react with biomolecules such as RNA, DNA, proteins, lipids etc., and change their normal physiology, resulting to development of complications like memory dysfunction [109, 155]. Previous reports suggests that oxidative stress can be alleviated by antioxidant treatment which in turns improve behavioral functions by improving neuronal survival and functions [155, 180, 181].

Despite well-known association of the hypoxia and oxidative stress with the onset of neurological complications, there is not even single drug available till date for the management or treatment of oxidative stress. Therefore, some alternative and safer treatment strategy is urgently needed which can alleviate oxidative stress during hypoxia and other conditions like diabetes, chronic stress etc. and rescue neurons from degenerations and associated complications. *S. chirata* and *S. cordata* are well known medicinal plants in Ayurveda, which are known for their beneficial effects during hepatitis, liver disorders,

inflammation, chronic fever, malaria, anemia, Gastrointestinal tract disorders, hypertension, mental disorders, and diabetes [1, 93]. Till date, numerous scientific studies have experimentally justified the traditional use of these plants for hepatic and other disorders [182], however, their use in the mental disorders remain unexplored so far, besides, the mechanisms through which the beneficial effects on brain are achieved remain unknown. Therefore, the present study was designed to get and insight to the neuromodulatory effect of *S. chirata* and *S. cordata* during hypoxia in Wistar rats and explore the oxidative stress as a potential mechanism.

6.2 MATERIAL AND METHODS

6.2.1 CHEMICALS AND REAGENTS

The chemicals and reagents used in this study were procured from Sigma, HiMedia, and Loba Chemie, unless otherwise specified. Kits for the measurement of oxidative stress parameters were procured from Roche Diagnostics (Roche Diagnostics K.K., Tokyo, Japan). UV-Spectrophotometric recordings were taken on nano-drop spectrophotometer (ND-2000C, Thermo Scientific). Medicinal plants were selected based on the traditional medicinal values of the plant, lack of experimental justifications to traditional use and ongoing extensive adulteration in the selected plant.

6.2.2 PLANT COLLECTION AND EXTRACT PREPARATION

S. chirata and *S. cordata* whole plants were collected between July and September from the high altitude regions of Himachal Pradesh and Uttarakhand. Both plants was identified by local herbal medical practitioners and authenticated by Prof. N. S. Chauhan, Dr. Y. S. Parmar University of Horticulture and Forestry, Solan, (H. P.) India. Plants were washed to remove dirt, shade dried at room temperature till constant weight was achieved and coarsely powdered. Plants were subjected to hydroalcoholic extraction (80:20; methanol: water) inside Soxhlet extractor at 25°C for 72 h. Extracts of both plants were filtered while hot. It was then concentrated by using rotary evaporator and lyophilized. Extract yield was calculated and the extract was stored at -20°C in a refrigerator until used for further analysis.

6.2.3 ANIMALS

Entire study was performed on male Wistar rats. Experimental procedures on the animals were approved by the Institutional Animal Ethics Committee (IAEC) (DIHAR/IAEC/ 36/2015). Experiments were performed strictly as per the guidelines of

CPCSEA, Govt. of India. Wistar rats (150-200g; 8-10 month age) were housed in the DIHAR animal house inside polypropylene cages (2 animals per cage) under standard conditions of temperature (23±2ºC), 12h day/light cycle and relative humidity (65±5%). Animals had free access to food and water.

6.2.4 ANIMAL GROUPINGS

Animals were classified into following groups.

Group 1- Control (CTRL) (received 0.3% carboxymethyl cellulose as vehicle orally)
Group 2- CTRL + 100 mg/kg *S. chirata* extract suspended in a vehicle (SCA 100)
Group 3- CTRL + 100 mg/kg *S. cordata* extract suspended in a vehicle (SCO 100)
Group 4- Hypoxia (HYP) (rats exposed to 10% oxygen for 3 days and received vehicle orally)
Group 5- HYP + 100 mg/kg *S. chirata* extract suspended in a vehicle (HYP-SCA 100)
Group 6- HYP + 100 mg/kg *S. cordata* extract suspended in a vehicle (HYP-SCO 100)

Each group had 5 animals and all the treatments were administered orally once daily between 6-7 pm for 7 days as per the groupings. Behavioral studies were performed 24 h after the last dose to avoid interference from the possible acute drug effect.

6.2.5 HYPOXIA INDUCTION

Animals were subjected to hypoxia inside a custom made hypoxia chamber. This chamber was linked to oxygen (O_2) and nitrogen (N_2) cylinders. The concentration of O_2 inside the chamber or the concentration of O_2 to which animals were exposed was regulated by N_2. In this study, hypoxia was inflicted by exposing rats to 10% O_2 for 3 days.

6.2.6 ROTA-ROD TEST

Effect of hypoxia and extract treatments on muscle coordination was evaluated through Rota rod test as per the method described by Mehta et al. (2017) [155]. Rota rod test is one of the most extensively used procedure to evaluate muscle coordination and strength in rodents. Animals were brought to the experimental room 30 min prior to the experimentation to get them acclimatize to the laboratory conditions. Each animal was placed on the rotating bar of the Rota rod (30 rpm) and time was recorded for each fall from the bar. Each animal was given three trials at an interval of 5 min with maximum cut-off time was 180 sec. The best time recorded was taken as the final reading to evaluate muscle coordination. To avoid

olfactory cues, the apparatus was wiped with 70% ethanol between every experimental session(s).

6.2.7 MEMORY FUNCTION TESTS

6.2.7.1 PASSIVE AVOIDANCE TEST

Passive avoidance paradigm was used to evaluate the effect of extract treatments on the hypoxia mediated memory dysfunction, the test was based on the method as described previously [155], with few modifications as per the requirements of our study. The apparatus consisted of two adjacent wooden chambers with one chamber painted white and was brightly illuminated by 100 W bulb placed 3 feet above the chamber and other chamber was painted black and covered to provide dark environment to the animals. Both chambers were interconnected through a small opening (5 cm × 5 cm), which provided free access to the animals to enter any chamber. 30 min prior to the start of learning trial, animals were brought to experimental room, lit with low intensity light. Each animal was first placed in the light chamber and entry to the dark chamber was recorded for each animal, with maximum cut-off or 180 sec. As the animal entered the dark chamber, an inescapable foot chock was delivered through grid floor. Animals were immediately removed and returned to their home cages. Memory retention was evaluated 24 h after the learning trials. Animals were again placed in the light chamber and time was registered with a maximum cut-off or 180 sec for entering the dark chamber. However, no foot shock has been applied this time. Entire device was cleaned between each experimental session with 70 percent ethanol to prevent any kind of olfactory cues.

6.2.7.2 ACTIVE AVOIDANCE TEST

Effect of hypoxia and extract treatments on memory acquisition and retention was evaluated through active avoidance paradigm as per the method described by Moscarello et al. (2013) [183], with few modifications as per the requirements of our study. The apparatus consisted of two adjacent wooden chambers painted white. Both chambers were having grid floor capable of delivering foot shock and were interconnected through a small opening (5 cm × 5 cm), which provided free access to the animals to enter any chamber. In one of the randomly selected chamber, animals were given scrambled electric foot while the other served as a non-shock chamber. Animals were brought to experimental room, illuminated by low intensity light, 30 min prior to the start of learning experiment. Each animal was placed

in the random chamber and were given conditioned stimulus through a buzzer for 2s. This was followed by a buzzer and foot shock of 40V and current 0.1 mA, for 4s. To avoid the foot shock, if rat moved to a shock-free chamber the avoidance was considered positive. If rat failed to move, it was gently guided to the shock-free chamber for earning. This process was repeated thrice and then animals were returned to their home cages. 24 h learning trials, animals were again brought to the experimentation room for memory retention trials. Each animal was placed into the random chamber of the apparatus and was given conditioned stimulus and the time taken to reach safe chamber was recorded as the avoidance behavior, with the maximum cut-off of 180 sec. No foot shock was given during the retention trials. The whole apparatus was wiped with 70% ethanol after every experimental session to avoid any possible olfactory cues.

6.2.8 ANIMAL SACRIFICE AND SAMPLE COLLECTION

For biochemical evaluation, animals were sacrificed by cervical dislocation, brain was dissected out, hippocampus was isolated, weighed, homogenized in HEPES buffers and stored at 4ºC until used. All the samples were used within 6 h for the biochemical evaluations. For histopathological examinations, animals were anesthetized with 90 mg/kg ketamine + 5 mg/kg Xylazine followed by fixation with 2% formaldehyde + 2% glutaraldehyde solution using double circulation technique. Tissues were collected and stored in fixing solution at room temperature until used further [155, 180].

6.2.9 MITOCHONDRIAL REACTIVE OXYGEN SPECIES (ROS) GENERATION

The effect of hypoxia and extract treatment on the generation of mitochondrial ROS was assessed with required modifications and according to the method outlined by Wasilewski and Wojtczak [154]. Briefly, Bradford method was used to quantify the amount of protein in the hippocampal homogenate. 15 µg protein were incubated with the reaction buffer (5 mM pyruvate, 2.5 mM malate, and 10 M of dichloro dihydrofluorescein diacetate) for 25 min at 37ºC. The level of fluorescence produced by the interaction of dichloro dihydrofluorescein diacetate with ROS was quantified using a 485 nm excitation spectrum Cary Eclipse fluorimeter (Varian, Palo Alto, USA) and a 582 nm emission wavelength. Results to protein content were standardized.

6.2.10 LEVELS OF LIPID PEROXIDATION

The effect of extract treatment on lipid peroxidation mediated by hypoxia has been tested with slight modification to the method reported by Mehta et. al. (2017a) [155]. Briefly, the hippocampal homogeneous equivalent of 15 µg protein was diluted with a 7.4 pH buffer of 0.1 M Tris-HCl. With constant mixing inside an incubator, the resulting mixture was incubated at 37°C for 2 h. One ml of trichloro acetic acid (10% w/v) was added to the reaction mixture and centrifuged at 10000 rpm. In addition, each tube was added 1.5 ml thiobarbituric acid (0.67 percent w / v), followed by incubation over a water bath maintained at 950°C for 10 min. The tubes were then kept at room temperature, centrifuged at 10,000 rpm for 15 min, supernatant was used to record absorbance at 532 nm using UV spectrophotometer. The results were expressed as nmol TBARS/mg protein.

6.2.11 CATALASE ACTIVITY

Effect of hypoxia and extract treatments on the hippocampal catalase activity was assayed as per the method described by Mehta et al. (2017a) [155]. Spectrophotometrically, catalase activity was recorded at 240 nm and the outcomes were expressed as moles of hydrogen peroxide (H_2O_2) consumed per minute. Hippocampal homogenate equivalent of 15 µg protein was diluted to 0.5 ml and 32 µl of 30% H_2O_2 was added to it. Absorbance was immediately recorded and readings were taken every minute for 4 minutes. The level of catalase in the hippocampal homogenate was determined in terms of the amount of H_2O_2 consumed during the 4-minute reaction interval.

6.2.12 GLUTATHIONE LEVELS

Method described by Necheles et al. (1969) [156], with minor modifications was used to determine effect of hypoxia and extract treatments on the hippocampal glutathione peroxidase activity. Reaction mixture was prepared by mixing 0.4 ml phosphate-buffer (0.35M; pH 7.4), 0.1 ml sodium azide (10mM), 0.2 µl freshly prepared glutathione (GSH) (8 mM), 0.5 ml hippocampal homogenate equivalent to 15 µg protein, 0.1 ml H_2O_2 (2.5 mM), and 1.1 ml double distilled water in respective tubes. These tubes were then incubated for 5 min at 37°C followed by addition of 0.5 ml trichloroacetic acid (10%). At room temperature, the reaction mixture was centrifuged at 3500 rpm for 15 min, supernatant was isolated, and 3 ml of disodium hydrogen phosphate (0.3 M) was used to dilute 0.5 ml supernatant. The reaction mixture was added to 1 ml of freshly prepared DTNB (phosphate buffer pH 7.4) and allowed the reaction mixture to stand at room temperature for 5 minutes. Using a UV

spectrophotometer, absorbance was registered at 412 nm and the outcomes were recorded as mg of GSH consumed / min / mg.

6.2.13 HISTOPATHOLOGICAL EXAMINATION

Effect of extract treatments on the hypoxia mediated neuronal damage was evaluated through histopathological examination, as per the method described previously [155, 180]. Briefly, brain of the animals were processed as described above. Fixed brain samples were embedded into the paraffin wax and thick section of 5 μm were prepared by microtome. Sections were fixed to glass slides, which were then stored at room temperature until used. Neuronal integrity and damage was detected by hematoxylin-eosin staining method. At 100X magnification, images of the sections were captured and the effect of hypoxia and extract treatments on neuronal morphology was determined.

6.2.14 STATISTICAL ANALYSIS

Using Graph Pad Prism 6, the statistical significance of the outcomes was evaluated. The values have been expressed as mean ± SEM. One way ANOVA and Dunnet post hoc test was used to determine the statistical significance at various of significance designated as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs control and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs HYP.

6.3 RESULTS

6.3.1 EXTRACTIVE YIELD AND PHYTOCHEMICAL EVALUATION

The crude extractive yield of the hydroalcoholic Soxhlet extraction of *S. chirata* and *S. cordata* was observed to be 22.37% and 26.32% respectively. We conducted qualitative phytochemical screening to get an idea of the phytochemicals in the extract, which revealed the existence of terpenoids, flavonoids, alkaloids, glycosides, steroids, tannins and saponins as the main components. Moreover, volatile oil, fixed oil, proteins and acidic compounds were not observed to be present in the plant extracts in significant amounts. We further observed that both extracts were having high amount of flavonoids and phenolic compounds which might be responsible for its antioxidant potential.

6.3.2 BEHAVIORAL INVESTIGATION

6.3.2.1 ROTA ROD TEST

Rota rod test was used to study the effect of hypoxia and extract treatments on muscle coordination and results are depicted in Fig. 6.1 (A). Subjecting animals to hypoxia resulted in a non-significant impairment in muscle coordination as the time taken to fall from the rotating bar was not significantly different than control animals. Likewise, no significant difference was observed in the muscle coordination of control and hypoxic animals which were treated with plant extracts, when compared to CTRL or HYP. These results suggest that short term hypoxia do not impair muscle coordination and animals had normal muscular activity.

6.3.2.2 PASSIVE AVOIDANCE TEST

Effect of *S. chirata* and *S. cordata* extract treatment on the hypoxia mediated memory dysfunction was evaluated through passive avoidance step-through paradigm and the results are depicted in Fig. 6.1 (B). Subjecting animals to hypoxia resulted in marked decrease in short term memory. The results of present study showed significantly ($p < 0.001$) reduced latency to enter the dark chamber during retention trials, thereby suggesting that during learning trials, animals were not able to remember the foot shock they received in the dark chamber. Pretreatment with plant extracts significantly improved memory performance in hypoxic animals, conveying that animals were able to remember the foot shock and were resistant to enter dark chamber. Animals preferred to stay in light chamber and the transfer latencies were significantly ($p < 0.01$) higher than HYP. Further, control animals treated with plant extracts were also able to retain foot shock memory and transfer latencies did not showed any deviations from CTRL.

6.3.2.3 ACTIVE AVOIDANCE TEST

Effect of *S. chirata* and *S. cordata* extract treatment on the hypoxia mediated learning and memory dysfunction was evaluated through active avoidance paradigm and the results are depicted in Fig. 6.1 (C). Hypoxia significantly impaired learning and memory in animals. Hypoxic animals were not able to learn the association between auditory cue and foot shock. These animals demonstrated significantly ($p < 0.01$) higher transfer latency to the safer region of the apparatus, suggesting impaired learning and memory. Extract treatment rescued animals from hypoxia mediated memory dysfunction. These animals efficiently remembered the association between audio cue and foot shock. The transfer latencies of these animals were significantly ($p < 0.01$) lower than the HYP animals. Moreover, control animals treated

with plant extracts also demonstrated good learning and memory functions and transfer latencies of these animals did not showed any deviations from CTRL animals.

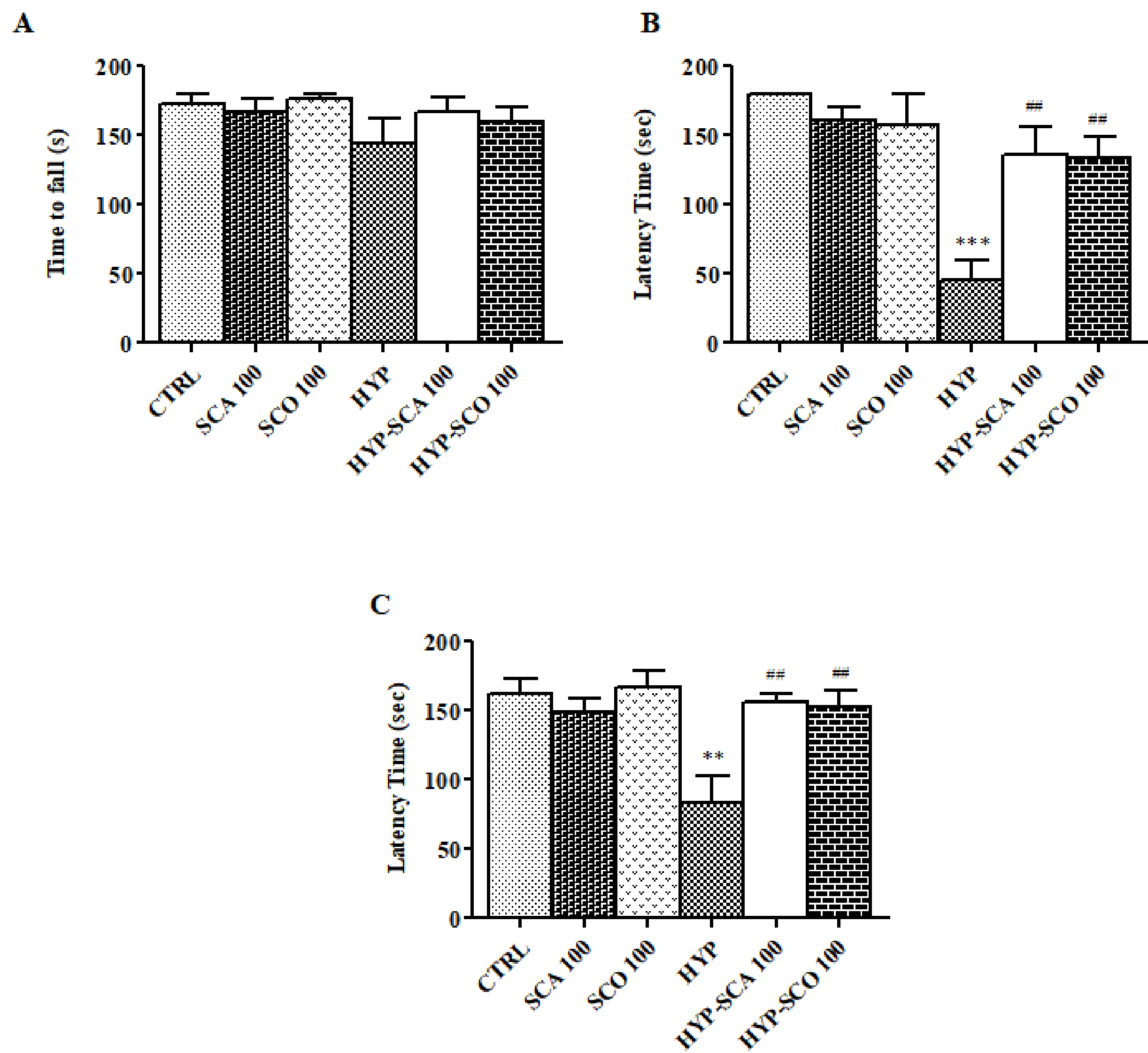


Fig. 6.1: Effect of *S. chirata* and *S. cordata* treatment on hypoxia mediated behavioral alterations (A- Rota-rod test; B- Passive avoidance test; C- Active avoidance test). Values are represented mean ± SEM. CTRL: control; SCA 100: control + 100 mg/kg *S. chirata* extract; SCO 100: control + 100 mg/kg *S. cordata* extract; HYP: hypoxia; HYP-SCA 100: HYP + 100 mg/kg *S. chirata* extract; HYP-SCO 100: HYP + 100 mg/kg *S. cordata* extract. ***p < 0.001, **p < 0.01, *p < 0.05, versus CTRL group.$^{###}$p < 0.00, $^{##}$p < 0.01, $^{#}$p < 0.05; versus HYP group.

6.3.3 BIOCHEMICAL INVESTIGATION

6.3.3.1 MITOCHONDRIAL REATIVE OXYGEN SPECIES (ROS) GENERATION

The results of the ROS generation in mitochondria and the effect *S. chirata* and *S. cordata* treatment are depicted in Fig. 6.2 (A). Hypoxia induced significant oxidative stress in the hippocampus and the levels of hippocampal ROS were observed to be significantly ($p < 0.001$) higher than the CTRL animals. The levels of 0.1 mA, ROS in control animals treated with plant extract did not showed any significant variations from the control animals, suggesting that extract treatment is not contributing directly to enhancing ROS production in hippocampus. Further, pretreating hypoxic rats with 100 mg/kg dose of *S. chirata* and *S. cordata* extract resulted in a significant ($p < 0.05$) reduction in mitochondrial ROS generation when compared to hypoxia treated rats. Our results suggest that both extracts are having comparable antioxidant potential, which may be attributed to high levels of flavonoids and phenolic compounds present in these plants.

6.3.3.2 LIPID PEROXIDATION

Lipid peroxidation is a measure of damage to the cellular membrane. The biochemical levels of malondialdehyde (MDA) are estimated as a marker of lipid peroxidation in the biological samples such as brain, especially in the animal experimentation. The results of hypoxia mediated lipid peroxidation and the effects of extract treatments are depicted in Fig. 6.2 (B). Our results suggest that hypoxia induced marked lipid peroxidation in the hippocampus, as the levels of MDA were observed to be significantly ($p < 0.001$) higher in HYP animals when compared to CTRL. Treating HYP animals with *S. chirata* and *S. cordata* extract reduced lipid peroxidation levels and the levels of MDA were observed to be significantly ($p < 0.001$) lower than HYP. Inhibition of lipid peroxidation was observed to be more pronounced in *S. chirata* treated animals than animals treated with *S. cordata* extract. Moreover, control animals treated with these extracts did not showed any significant difference when compared to CRTL, eliminating any possibility of acute effect of plant extracts.

6.3.3.3 CATALASE ACTIVITY

Catalase is one of the most potent antioxidant enzymes present in our entire body, including brain. It is actively involved in the detoxification of ROS generated during the functioning of electron transport chain. The results of effect of hypoxia and extract treatments in hippocampal catalase levels are depicted in Fig. 6.2 (C). Subjecting animals to hypoxia resulted in the depletion of hippocampal catalase levels. Catalase levels were observed to be significantly ($p< 0.001$) lower in HYP animals, compared to CTRL animals. Pretreatment of hypoxic animals with plant extracts improved hippocampal catalase levels significantly ($p<0.01$) when compared to HYP untreated animals. Animals treated with plant extract only did not demonstrated any significant deviation in catalase levels, when compared to CTRL, eliminating any possibility of direct effect of plant extracts. Our results suggest that *S. chirata* and *S. cordata* extracts are having a good antioxidant potential which can be partially attributed to its potential to either upregulate catalase activity or prevent its depletion during oxidative stress.

6.3.3.4 GLUTATHIONE LEVEL

The effect of hypoxia and extract treatment on hippocampal glutathione levels was assessed and the results are depicted in Fig. 6.2 (D). Subjecting animals to hypoxia resulted in a significant ($p <0.01$) depletion or lowering of glutathione levels in the hippocampus region of the brain. The results of current study demonstrated a comparable and marked antioxidant potential of extract treatments. Hippocampal glutathione levels were significantly ($p < 0.05$) improved after treating hypoxic animals with *S. chirata* and *S. cordata* extracts, as compared to HYP. As compared to CTRL the normal animals treated with extract did not showed any significant difference in hippocampal glutathione levels, and thereby suggesting observed effects of treatments during hypoxic conditions are the result of rescuing hippocampus form hypoxia mediated damage.

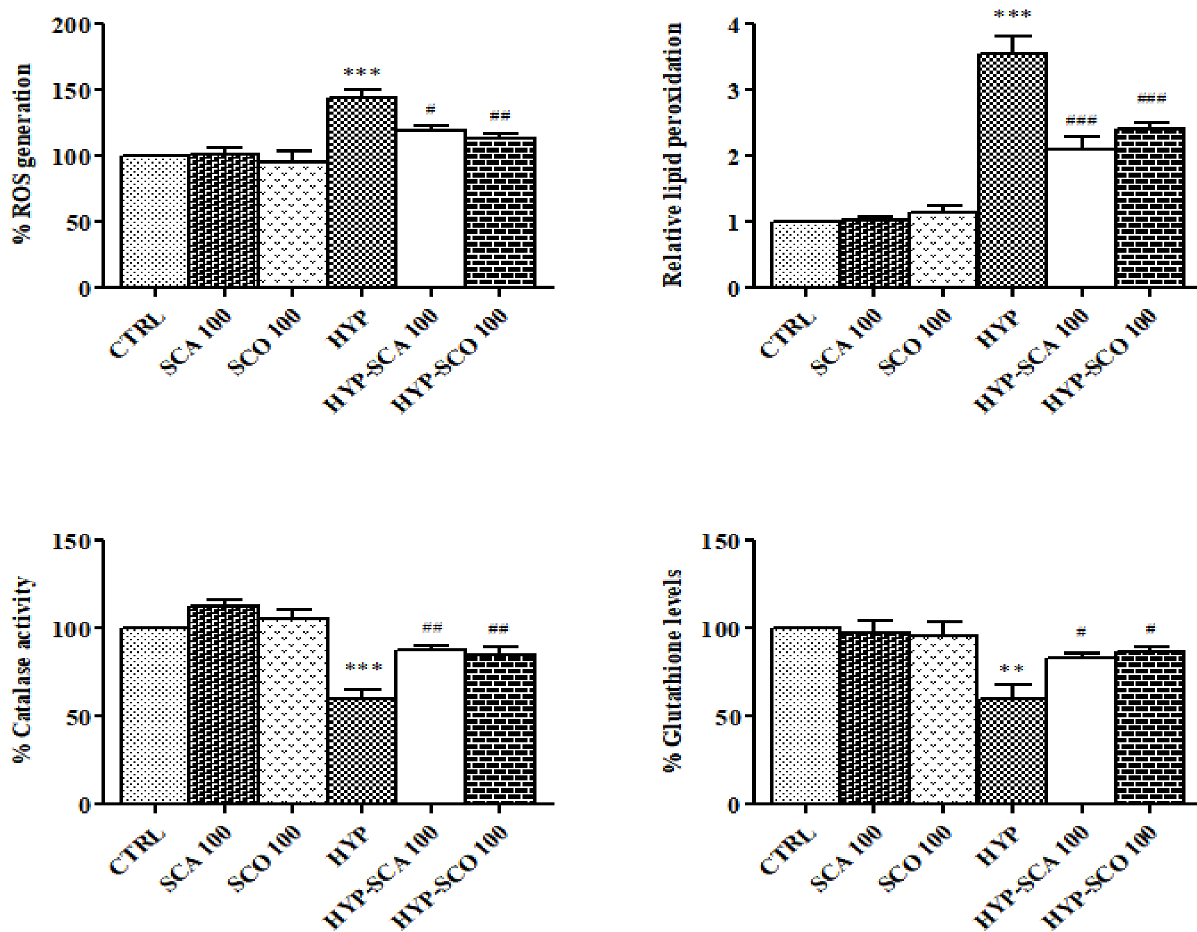


Fig. 6.2: Effect of *S. chirata* and *S. cordata* treatment on hypoxia induced oxidative stress (A- % ROS generation; B- lipid peroxidation; C- % catalase activity; D- % glutathione levels). Values are represented mean ± SEM. CTRL: control; SCA 100: control + 100 mg/kg *S. chirata* extract; SCO 100: control + 100 mg/kg *S. cordata* extract; HYP: hypoxia; HYP-SCA 100: HYP + 100 mg/kg *S. chirata* extract; HYP-SCO 100: HYP + 100 mg/kg *S. cordata* extract. ***p< 0.001, **p< 0.01,*p< 0.05; versus CTRL group. $^{###}$p< 0.001, $^{##}$p< 0.01 $^{#}$p< 0.05; versus HYP group.

6.3.4 HISTOPATHOLOGICAL EXAMINATION

Hematoxylin-eosin staining is widely used method to determine the morphology of neurons or neuronal damage. In the present study this technique was used to establish the effect of hypoxia on the neuronal morphology and also to evaluate the neuroprotective effect of *S. chirata* and *S. cordata* extract treatments. Results are depicted in Fig. 6.3. Analysis of

the stained sections demonstrated the normal neuronal morphology in samples belonging to CTRL animals Fig 6.3 (A). Hypoxia induction resulted in marked neuronal damage and neurons appeared to be degenerated or damaged Fig 6.3 (D). Plant extract pretreatments protected neurons from hypoxia mediated damage and when compared with CTRL, the neurons of these animals had similar normal morphology with minor signs of damage and degeneration Fig. 6.3 (B, C). These results concluded that *S. chirata* and *S. cordata* extract can protect neurons from hypoxia mediated damage, which might have resulted in improved memory functions in these animals.

6.4 DISCUSSION

The present investigation demonstrated the prevention of memory impairment in hypoxia treated rats by using plant extracts of *S. chirata* and *S. cordata* treatment against hypoxia in Wistar rats. Hypoxia leads to the generation of excessive free radicals that exceed the neutralizing ability of the body's antioxidants, such as SOD, glutathione, catalase, etc. [109, 155]. These free radicals then circulate in the body and damage the functioning of essential biomolecules causing neurological problems such as depression (Anderson & Maes 2014) [184], cognitive dysfunction [185], Alzheimer's disorder [159], dementia [159], hepatotoxicity [184], cellular damage [157], necrosis [157], apoptosis [158] etc. Moreover, these free radicals induce interact with the membrane phospholipids and induces lipid peroxidation, which further result in the cellular damage and degeneration [109, 110, 155]. Previous reports suggest that the hypoxia markedly impaire learning and memory functions in rodents [186, 187]. Our results are in line with these reports. Herein we demonstrate marked impairement in learning and memory in hypoxic wistar rats during active avoidance and passive avoidance paradigm. Moreover, we observed high levels of ROS and lipid peroxidation with depleted catalase and glutathione in the hippocampus of these animals, which is primarily associated with the memory and lerning functioning in rodents. Many studies associate the development of behavior dysfinction with the enhanced oxidative stress [188-190] and alleviation of oxidative stress is demonstrated to result in improved behavior in animals [155, 189, 191, 192]. We further demonstrated that treating hypoxic animals with *S. chirata* and *S. cordata* extracts reduced ROS generation and lipid peroxidation in the hippocampus and enhanced antioxidant levels in rat brain. We further reported the presence of high levels of phenolic compounds and flavonoids in the hydroalcoholic extracts of these

plants. Phenolic and flavonoids are potent antioxidants and literatures report the neuromodulatory effect of herbal preparations which are rich in these antioxidants [193-195]. In our study, these plant extracts significantly improved learning and memory, which may be the result of alleviation of the hippocampal oxidative stress. No significant difference was observed in the muscle coordination of various treatment groups and thereby any possibility of increased transfer latency in memory test as a result of muscular insufficiency is eliminated. Further, histopathological examination of the rat brain revealed that hypoxia induced marked degeneration in the brain, which may probably be due to the enhanced oxidative stress and may be a crucial contributing factor in memory dysfunction. Previous reports suggest that neurodegeneration and neuronal damage are associated with impaired memory function in rodents [155, 181], besides indicating enhanced oxidative stress as an important contributing factor for neurodegeneration [155, 181]. Neuroprotective potential of herbal interventions have been reported to be associated with the improved behavior functions [155, 181, 193, 195]. Our results were observed to be similar with these findings and the present study was able to demonstrate that treatment with plant extract improve neuronal morphology and protect hypoxic animals from degenerative changes. These results suggest that hydroalcoholic extract of *S. chirata* and *S. cordata* efficiently improved hypoxia mediated memory dysfunction which can be attributed to their potential to alleviate hypoxia-induced oxidative stress in the brain.

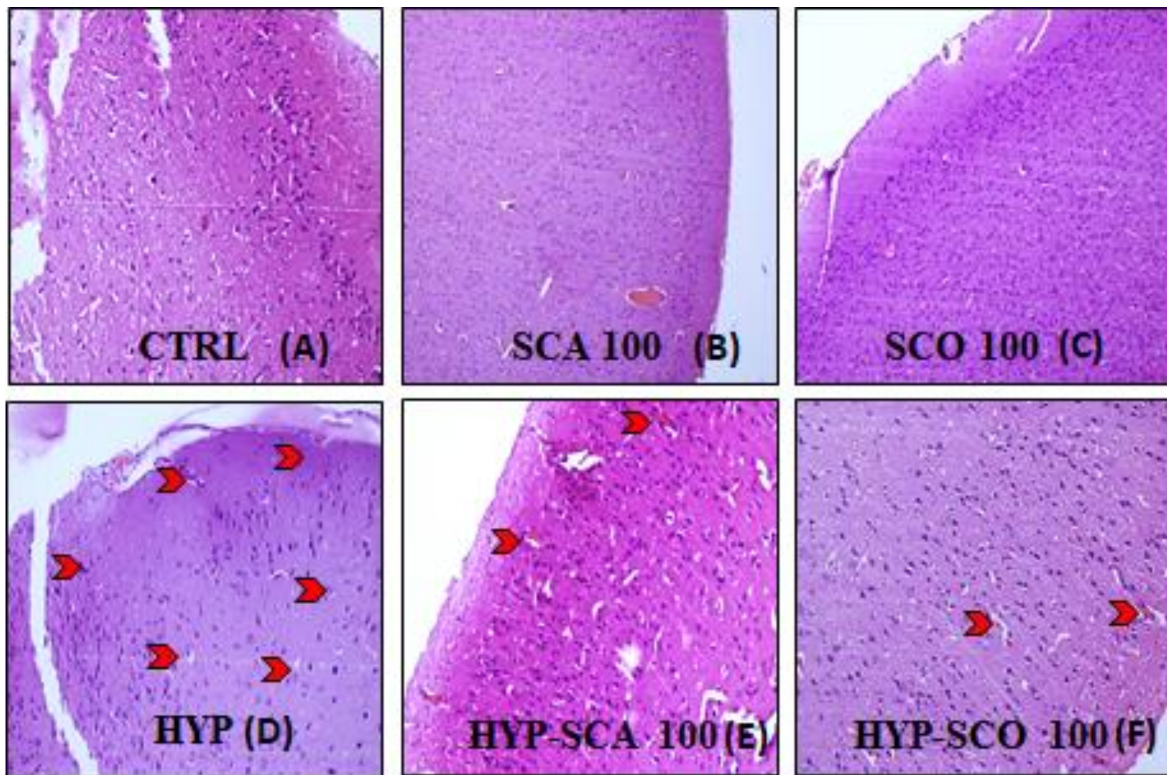


Fig. 6.3: Effect of *S. chirata* and *S. cordata* treatment on hypoxia induced neuronal damage. Histopathological examination of brain sections using hematoxylin-eosin staining. Red arrow indicate the damage regions on the histopathological sections.

6.5 CONCLUSION

Hypoxia is associated with enhanced oxidative stress, neuronal damage, and the development of memory dysfunction in Wistar rats. The hydroalcoholic extract of *S. chirata* and *S. cordata* prevented memory dysfunction and demonstrated comparable neuroprotective potential which was linked with the alleviation of hypoxia-mediated oxidative stress. Our finding provides an experimental justification for the traditional use of these plants during brain disorders. Moreover, herbal preparations are safe and may have a clinical application for the management of oxidative stress-related neurological complications. These findings are preliminary and need to be evaluated further.

OVERALL CONCLUSION
AND
FUTURE DIRECTIVES

Our findings suggest that the psbK-I locus can be efficiently exploited for differentiating various *Swertia* species and their adulterants. Moreover, we have demonstrated that the DNA bar coding may prove to be very important tool for authentication, validation, identification and classification of different species. Based on the outcome of our study, we further suggest that DNA barcoding technology may be applied for resolving complicated issues regarding identification and discrimination problems associated with *Swertia* species and their adulterants. Moreover, our findings suggest that Tetradecanal, Methyl 3-hydroxy-2-oxobutanoate, 1,2-benzenedicarboxylic acid as the most potential marker compounds to differentiate *S. chirata* from its adulterants. These metabolites can be used to differentiate the quality and identify adulteration of *S. chirata* samples by utilizing technique of Phylogenetic analysis based on GC-MS.

Second part of the thesis focus of the medical value of the *S. chirata* extract and its comparison to its most common adulterant so as to differentiate the efficacy of drug from the adulterants and to provide a scientific justification to the traditional medicinal value of *S. chirata*. For this, hypoxia model was used that was observed to be associated with the development of hepatic damage, enhanced oxidative stress, altered hepatic morphology and disrupted hepatic biochemical profile in Wistar rats. The hydroalcoholic extract of *S. chirata* demonstrated marked hepatoprotective potential which was associated with the attenuation of hypoxia-mediated oxidative stress. Our finding provides an experimental justification for the traditional use of this plant during the liver disorders. Further, hypoxia is associated with enhanced oxidative stress, neuronal damage, and the development of memory dysfunction in Wistar rats. The hydroalcoholic extract of *S. chirata* and *S. cordata* prevented memory dysfunction and demonstrated comparable neuroprotective potential which was linked with the alleviation of hypoxia-mediated oxidative stress. It is to be noted that the results for the *S. chirata* were far better than those observed for the *S. cordata*. Our finding provides an experimental justification for the traditional use of these plants during brain disorders.

However, these results are preliminary findings and need extensive experimental validation as a future prospects. Moreover, the use of barcoding technique to differentiate herbal drug from its adulterants could prove to very crucial based on the degree of accuracy and cost effectiveness of this technique, when compared to the currently available techniques used to check adulterants in herbal drugs. This technique could also be validated for other herbal drugs.

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