DNA BARCODING ANDPHYLOGENETIC ANALYSIS OF *RHODODENDRON SPP* AND PHYTOCHEMICAL ANALYSIS OF *EPHEDRA GERARDIANA* (WALL.EX STAPF) FROM SAGARMATHA NATIONAL PARK, NEPAL

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Rajgir Mahato

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TO WHOM IT MAY CONCERN

Mr.RajgirMahato, who enrolled in Masters of Science in Central Department of Biotechnology (CDBt), Tribhuvan University (TU), Kathmandu, Nepal, successfully conducted his M.Sc. dissertation research works on **"DNA BARCODING AND PHYLOGENETIC ANALYSIS OF *RHODODENDRON SPP* AND PHYTOCHEMICAL ANALYSIS OF *EPHEDRA GERARDIANA* (WALL.EX STAPF) FROM SAGARMATHA NATIONAL PARK, NEPAL"** at our laboratory for the partial fulfillment of his academic program. The work conducted by Mr. RajgirMahato was jointly supervised by us and his supervisor Prof. RajaniMalla from CDBt, TU. As external supervisors, we wish him all the best for the successful submission of his thesis.

……..	……
Dr. Sangita Shrestha	**Dr. GanBahadurBajracharya**
Supervisor	**Supervisor**
Chief Scientific Officer and Unit Chief	Senior Scientific Officer
Molecular Biotechnology Unit	Natural Product Chemistry Unit
Nepal Academy of Science and Technology (NAST)	Nepal Academy of Science and Technology (NAST)
Khumaltar, Lalitpur, Nepal	Khumaltar, Lalitpur, Nepal

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LIST OF ABBREVIATIONS

µg	micro gram
µl	micro litre
ADP	Adenosine Diphosphate
AFLP	Amplified Fragment Length Polymorphism.
AGE	Agarose Gel Electrophoresis
APG	Angiosperm Phylogeny Group
AP-PCR	Arbitarily Primed Polymerase Chain Reaction
ATCC	American Type Culture Collection
BF	Bayesian Factors
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Data System
BSLA	Brine Shrimp Lethality Assay
CAPS	Cleaved Amplified Polymorphic Sequence.
CBOL	Consortium for Barcode of Life
CI	Consistency Index
CITES	Conservation of International Trade in Endangered
cDNA	Complementary DNA
CNS	Central Nervous System
COI	Cytochrome c Oxidase I gene
CpDNA	Chloroplast DNA
CFU	Colony Forming Unit
CTAB	Cetyltrimethyl Ammonium Bromide
DAF	DNA Amplification Fingerprinting
ddNTP	dideoxy Nucleoside Triphosphate
DMSO	Dimethyl SulphoOxide
DNA	Deoxyribonucleic Acid
dNTP	deoxy Nucleoside Triphosphate
DPR	Department of Plant Resource
EB	Ethidium Bromide
EBC	Everest Base Camp

FDA	Food and Drug Administration
GTR+I	General Time Reversal with Invariable sites
HKY	Hasegawa, Kishino and Yano
ITS	Internal Transcribed Spacer
ISSR	Inter-Simple Sequence Repeats.
JC	Jukes and Cantor
K2P	Kimura 2- Parameter
LC50	Lethal Concentration 50
MAPs	Medicinal and Aromatic Plants
matK	Maturase K
MEGA	Molecular Evolutionary Genetic Analysis
mg	milli gram
MHA	Muller Hilton Agar
mL	milli litre
mm	milli motor
mM	milli Molar
MP	Maximum Parsimony
NAST	Nepal Academy of Science and Technology
NCBI	National Center for Biotechnology Information
NTFPs	Non Timber Forest Products
OUT	Operational Taxonomic Unit
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
rbcL	Ribulose-1,5-bisphosphate carboxylase- Large subunit
rDNA	ribosomal Deoxyribonucleic Acid
RFLP	Restriction Fragment Length Polymorphism.
RI	Retention Index
SCAR	Sequence Characterized Amplified Region
SNP	Sagarmatha National Park
SPR	Subtree- Pruning- Regrafting
STRs	Short Tandem Repeats
STMs	Sequence Tagged Microsatellites

SSLP	Simple Sequence Length Polymorphism
SSR	Simple Sequence Repeats
TCM	Traditional Chinese Medicine
TE	Tris- EDTA
TPM1uf	Kimura-3- Parameter with unequal frequenc
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
ZOI	Zone Of Inhibition

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ABSTRACT

DNA barcoding was performed using three chloroplast DNA (cp DNA) loci, *viz*, *matK, rbcL* and *trnH-psbA* and one nuclear DNA loci the Internal transcribed spacer (nrITS). Variable sites for *matK, rbcL, trnH-psbA* and ITS loci was found to have 435, 172, 278, and 533 respectively. Phylogenetic analyses were performed using model averaged phylogeny (jModelTest 2.1.4) and parsimony based method (MEGA 6.0). The best fitted nucleotide substitution model was computed based on corrected Bayesian Information Criteria (BIC) using jModelTest and was found to be HKY+G for *matK,* HKY+I for *rbcL,* TPM1uf for *trnH-psbA* and K80+G for ITS. Maximum Parsimony (MP) trees were constructed for all loci using MEGA 6.0 found to be Consistency index (CI) value 1.000 for *matK* and *rbcL* while 0.951 for *trnH-psbA*. Similarly, CI for ITS found to be 0.984.*matK* sequences were able to distinguish the Rhododendrons sequences from outgroup sequences and placed all the accessions in single clade. In the Maximum parsimony (MP) tree, outgroup species formed separate clade and *R. nivale* was shown sister species to them. Other Rhododendron spp. formed ploytomy in the cladogram.This showed that based on *rbcL* all Rhododendron spp. under study formed polytomy and were not further differentiated into specific sub-clade. NCBI accession *R. simsii* is shown to be sister species of two outgroup accsessions. In case of, trnH-psbA, all accession species and two*Gaultheria spp.* taken as outgroup seuences were placed in two clade and shown the same sister species pattern similar to that of strict consensus tree generated using jModelTest thus indicated the validity of result obtained. Thus, jmodel and MP shown the highly congruent cladogram. In case of ITS, *R. arboreum* shown to be sister species of outgroup. Monophyletic clade consisting *R. setosum, R. anthopogon, R. nivale*&*R. lepidotum* are seen in both the cladogram. Hence these jModel shown partially congruent with MP tree.All of the barcode resolved all the species almost into a monophyletic clade so it can be potential barcode combined with other barcode.Methanolic phytochemical yielded higher amount of extract as compared to hexane extract of plants. Phytochemical screening of *Ephedra gerardiana* shown it contains various phytochemical consituents. Antibacterial activity of both hexane and methanol extract of *E. gerardiana* was assessed and was found that these bacterial strains were resistant to the plant extracts. LC_{50} values were computed for extracts, 45.71 for methanol and 117.49 for hexane, methanol extract is more toxic than hexane extract.

Keywords:*Rhododendron spp., Ephedra gerardiana*, DNA barcode, *matK, rbcL,trnH-psbA*, ITS

1.1 Introduction

Nepal is a small, landlocked country situated between two giant countries India and China. It lies on southern slope of central Himalaya and occupies a total area of 1,47,181 sq. km between the latitude of 26°22' and 30°27' N and the longitude of 80° 40' and 88° 12' E. The average length of the country is 885 km from east to west and north to south width variedfrom 145 km to 241 km. Nepal is blessed with varied soil, topography and climatic conditions suitable for the growth of diverse plant species. The indigenous people of Nepal from different ethnic communities are well acquainted with the properties and uses of plants from their surroundings. More than 75% Nepalese people still depend on the herbal plants as a local source of medicine for their primary health care (Dutta, 2007). Until the middle of the 19th century, plants were the main therapeutic agents used by humans. About 60% of the world population and 60-90% of the population of developing countries rely on traditional medicine and about 85% of the traditional remedies for primary health care are derived from plants.

Nepal has been regarded as "the Natural Showroom of Biodiversity" because of its geographical, ecological and climatic variation. It comprises 0.1% of the earth's land area yet it ranks within the first quartile for global biodiversity importance (BPP, 1995). This is because of its unique bio-geographic location, altitudinal variation and diverse climatic and topographic conditions. It has 35 forests types, 75 vegetation units and 118 ecosystems (Staintan, 1972, Gyawali, *et al* 2008; DPR, 2012). Nepal is ranked as 9th among the Asian countries for its floral wealth with an estimated 9,000 species of flowering plants (Bhattarai *et al.*, 2013). So far around 6,653 species have been reported (Press *et al*, 2000, Kunwar *et al.*, 2010). It is estimated that there are about 1950 species of medicinal and aromatic plants in Nepal (Ghimire *et al.*, 2008).

Medicinal and Aromatic Plants (MAPs) possesses medicinal properties and hence are being employed by different medical symptoms to cure various ailments. MAPs are the main constituents of many traditional medicines like Ayurveda, Chinese and Tibetan Medicine, Homeopathy, Unani etc. A variety of medical drugs have been developed from different medicinal plants of Nepalese origin. Traditional medicine is used extensively by majority of the population of Nepal in the form of Ayurvedic, Traditional Chinese medicine (TCM), Unani, Tibeten, Amchi and homeopathy etc.Traditional medicine in Nepal comprises those practices that are based on beliefs which were in existence for hundreds to thousands of years

before the development and spread of modern medicine, and which are still in use today. In the past, many rural areas of Nepal, traditional medicinal knowledge and practice were passed down from generation to generation entirely via verbal mode of transmission and personal experience (Katiyar *et al.*, 2012).Medicinal plants play vital roles in the Nepalese livelihood and studies have shown that substantialproportion of Nepalese population from rural areas depend heavily on collection and trade of MAPs (Edwards, 1996). Every year an average of 20,000 tons of raw medicinal plants, worth between 8.6to 27 million US dollars is exported from Nepal to many countries of the world including India, China, Europe, the United States etc (Edwards, 1996, Subedi, 1993).

Nepal is very rich in *Rhododendron spp* and many of them have various economic values. They are distributed from east to west of Nepal. However, they are more concentrated in the areas between the Arun valley and Sikkimese border in eastern Nepal. *Rhododendron spp* are found from subtropical (*R. arboreum*) to nival regions (*R. nivale*) of Nepal with the highest number of species in subalpine zone (Milleville, 2002). Thirty onespecies of *Rhododendronspp*have so far been reported in Nepal (Press *et al.*, 2000; Rajbhandari & Watson 2005). *Rhododendron spp* are one of the most frequent and magnificent group of flowering plants in Sagarmatha National Park (SNP). Out of 31 species, 14 species are found in SNP, six species are found throughout the Nepal while other six species are found only in eastern Nepal (Rajbhandari & Watson, 2005). They are the most frequent plant species among the NTFPs of SNP (Bhattarai *et al.*, 2009). The species found in SNP are *Rhododendron anthopogon*D. Don, *Rhododendron arboreum*Sm, *Rhododendron barbatum*Wall ex G. Don, *Rhododendron campanulatum*D. Don, *Rhododendron nivale*Hook. f., *Rhododendron lepidotum*Wall ex G. Don, *Rhododendron setosum*D. Don, *R. triflorum, and R.hodgsonii*Hook.f.. In addition to these species, occurrence of *R. cinnabarium* has been reported by Bhuju *et al*. (2007) and *R. hodgsonii* and *R. wallichii* by de Milleville (2002), *R. lindleyi* and *R. dalhousiae* by Rajbhandari & Watson, (2005).Eight species of the *Rhododendron* in Nepal are reported to have medicinal and aromatic uses (Baral & Kurmi 2008). Given the socioeconomic, cultural, and ecological importance, the need of conservation of *Rhododendron* spp. has been urged in Nepal (Shrestha, 1999) and elsewhere (Kuniyal, 2002).

1.2 Research hypothesis

H_0: There is no nucleotide sequence variation in all tested loci of different species of Rhododendron of Sagarmatha National park, Nepal

H_o: There is no effective of Ephedra gerardiana on pathogens and Brine- shrimp

H_1: There is nucleotide sequence variation in all tested species of Rhododendrons of Sagarmatha Natrional Park, Nepal.

H_1: There is effective of Ephedra gerardiana on pathogens and Brine- Shrimp

H_0: Null Hypothesis; H_1: Alternate Hypothesis

1.3 Objectives

The overall objectives of this study is to characterize Rhododendronspp. of Sagarmatha National Park at molecular level by using sequence based DNA barcoding technique and chemical characterization of *Ephedra gerardiana.*Specific objectives of this project are:

1. Toamplify*ITS, matK, rbcl, trnH-psbA* sequences for DNA barcoding of *Rhododendron spp.* of SNP region.
2. To use the sequence information for species identification and interspecific phylogenetic analysis of *Rhododendrons spp.* of SNP.
3. To conduct phytochemical screening, antimicrobial and cytotoxicity bioassay of the *Ephedra gerardiana* of SNP.

1.4 Justification of the Study

The valuable Medicinal and Aromatic plant biodiversity of Nepal is under constant threat from various anthropogenic activities such as deforestation, land degradation, overexploitation as well as prevailing climatic change or global warming.

In this context, biodiversity documentation at molecular level holds great promise as it reveals intrinsic genetic variations at species and population levels that in turn furnish valuable information for the formulation of effective conservation strategy by the government. There is high chance that new species are discovered by such studies as study

based on DNA sequences (i.e. DNA barcoding) & phylogenetic analysis generate direct evidence of genetic variation and relatedness existing among different populations of a single species or among different species. Furthermore, DNA barcoding is crucial for positive identification of species and hence helps minimize illegal trades of high value MAPs of Nepal.

1.5Scope of the study

Medicinal and Aromatic plants are richest source of economy for the whole country, however various anthropogenic activities including over exploitation for trade has rendered many such plants vulnerable to extinction. Thus, this study has openeda new avenues for research not only in Rhododendrons but also in many other rare, threatened and vulnerable medicinal plants species of Nepal. DNA Barcoding technology has been recently visualized as an effective tool for species identification and discovering new or cryptic species and patenting of products and process pertinent to these valuable plants. Besides the general use of DNA barcoding technique in species identification, it has been very helpful in identifying adulterants of closely related medicinal plant species. Barcoding studies of most if not all medicinal plants of Nepal can someday furnish valuable data and methods to rapidly identify medicinal plants banned for trade and collection thus aiding enforcement of national conservation law. Furthermore, this study urged a larger study involving all species of Rhododendron found in Nepal.Study of antimicrobial and properly cytotoxicity along with screening of active compounds can aids in development of potential new and effective allopathic drugs and ayurvedic formulations particularly aimed at treating specific diseased conditions.

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CHAPTER 2: LITERATURE REVIEW

2.1TheFamily Ericaceae:

The APG III system of classification (Hasto *et al.,* 2009) (unchanged from APG I system of 1998 and APG II system (Haston *et al.,* 2003) recognizes the family Ericaceae and places it in the class Mangoliopsida and order Ericales in the clade eudicots (APG, 2009).This family is a family of flowering plants, commonly known as the heath or heather family, found most commonly in acid and infertile growing conditions. The family is large, with roughly 4000 species spread across 126 genera, making it the 14th-most-speciesrich family of flowering plants. The many well-known and economically important members of the Ericaceae include thecranberry, blueberry,huckleberry,azalea, rhododendron, and various common heaths and heathers(for example *Erica, Cassiope,Daboecia and callina*)(Internet visit first, 2014).

Plants of this family are evergreen or sometimes deciduous trees or shrubs, occasionally epiphytic; indumentum of simple or compound hairs, peltate scales, or glabrous. Componds hair type include; stellate (star like, arms rigid, spreading from apex of a short to long stalk); dendroid (tree like, arms flexuous, arising from apex of a long stalk); capitellate (dendroid, but with a short broad stalk); or fasciculate (hairs clustered, dendroid but with no stalk). Leaves simple, alternate or clustered at branch ends, usually coriaceous, entire.Flowers in mostly terminal (occasionally axillary) condensed racemes, rarely solitary, slightly (occasionally strongly) Zygomorphic.Calyx 5(-10) - lobed, often reduced to shallow cup or rim, persistent in fruit.Corolla campanulate, funnel- shaped or salver – shaped (tubular with spreading lobes), rarely cylindric, lobes 5- 10, long or short. Stamens usually twice as many as the corolla – lobes (sometimes more numerous, occasionally fewer); filaments without appendages; anthers without appendages, dehiscing by apical pores, pollen dispersed in strings. Carpels 5, fused; ovary superior, 5- 18 – celled, with hairs or scales, rarely glabrous; style terminal, persistent in fruit; stigma capitate, creante or lobes. Fruit a woody, cylindrical to ovoid septicidal capsule 4- 18- valved, dehiscent from top, persistent; seeds numerous, minute, fusiform, often winged or tailed (Rajbhandari & Watson, 2005).

2.2Distribution of Rhododendrons

2.2.1 World distribution

Over 4000 reported spp. ofrhododendrons are distributed in number of humid and cool regions across the northern hemisphere. These plants generally do not grow at low altitudes, preferring mountainous areas that have a temperate climate, although there are some that do flourish in alpine conditions (Mierow, 1978). Rhododendrons reach their most significant development in high hills and mountains that have two clearly different seasons, one dry and one rainy, as well as rather cool summers. These conditions are found at their best in the area extending from eastern Nepal to Yunnan province of China between elevations of 2000 and 4000 m (Milleville, 2002).

The Rhododendron's natural habitats extends further towards Southern China as well as to northeastern China, India, Bhutan and Japan. A good number of them are seen in the Burmese mountains and in the high hills surrounding Chiang Mai in Thailand (Polunin et al. 1984). The sufficiently cool hills in the otherwise tropical Malaysia, Borneo, Sumatra, Java, and New Guinea, is home to quite an important group known as Malaysian Rhododendrons (Milleville, 2002) The Bhutan ad Sikkim is covered by 70 percent of the Rhododendrons where as in Nepal the forest cover with Rhododendrons is only 35 percent of(Pradhan *et al.*, 1990)

2.2.2 Distribution in Nepal

In the area between the Arun valley and the Sikkimese border in eastern Nepal, the number of rainy days-including the mansoons and localised rains reaches 160 mm in a year on comparison to only 90 mm in the far west. Rhodoendrons require a fair amount of humidity for good growth. That is why there are many more varieties of Rhododendrons in eastern Nepal (Milleville, 2002).

The climate of Nepal is not much different from Sikkim and Darjeeling.However, the north-south kangchenjunga barrier and its southern extension, the Singalila Range, seems to act as an obstacle for the propagation of Rhododendrons. This explains the occurrence of 84 species in Sikkim, a number that goes down to 30 for whole of Nepal, just across the Singalila.A second limit is formed by longitude 87 ^{o}east as is evident from the fact that 25 species are

found in the Makalu- Barun National Park, but the nearby Sagarmatha National Park of Khumbu has only 10 species.Between these parks lies another high mountain barrier that sticks out south at exactly 87 ^{o}E-from Lhotse (8516 m) to Baruntse(7129 m) and Chamlang (7319 m).The number of species goes down progressively as one moves to the west – 11 species in the Lantang- Gosainkund region in central Nepal but only five in western and far – western Nepal.

Table2.1. Distribution of Rhododendron species found in Nepal.

Rhododendron species	**Habit**	**Distribution**	**Altitudinal Range(m asl)**
R.anthopogon	Shrub	Kashmir to southeast Tibet; far-western Nepal	3000-4800
R.arboreum	Tree	Kashmir to southeast Tibet and even Sri Lanka; all over Nepal	1500-3600
R.barbatum	Tree	Kumaon to Bhutan; all over Nepal-frequently dominant around 300 m in central Nepal	2400-3600
R.campanulatum	Shrub	Kashmir to southeast Tibet; all over Nepal	3000-4000
R.campylocarpum	Shrub	East Nepal to southeast Tibet	3300-4000
R.hodgsonii	Shrub	East Nepal to southeast Tibet	3000-3800
R.lepidotum	Shrub	North Pakistan to southeast Tibet, sechuan and North Yunnan;all over Nepal	2400-4500
R.nivale	Shrub	Nepal to southeast Tibet;in Nepal all over the country	4500-5500
R.setosum	Shrub	Central Nepal to southeast Tibet; in Nepal much abundant in khumbu region	3500-4800
R.camelliflorum	bush	East Nepal to southeast Tibet	2800-3000
R.ciliatum	Shrub	East Nepal to southeast Tibet	2700- 3900

R.cinnabarinum	Shrub	East Nepal to southest Tibet	3000-3600
R.cowanianum	Shrub	Endemic to central Nepal	3000- 4000
R.dalhousiae	Shrub	Central Nepal to southest Tibet	2000- 2700
R.falconeri	Tree	Far- eastern Nepal	2400-3400
R.fulgens	Shrub	Far-eastern Nepal to southeast Tibet	3300- 4200
R.glaucophyllum	Bush	East Nepal to southeat Tibet, rare in Nepal	2700-3600
R.grande	Tree	East Nepal to southest Tibet	1700-3300
R.griffithianum	Tree	East Nepal to southeast Tibet	2100-3000
R.lindleyi	Shrub	East Nepal to southeast Tibet	2000- 3300
R.lowndesii	Bush	Endemic to central Nepal and eastern Nepal.	3200- 4500
R.pendulum	Bush	East Nepal to southest Tibet	2500- 3600
R.pumilum	Shrub	East Nepal to Yunnan, rare in Nepal	3500- 4300
R.thomsonii	Tree	East Nepal to southest Tibet	3000- 3800
R.trichocladum	Bush	East Nepal to Yunnan, rare in Nepal	2700-3300
R.triflorum	Shrub	East Nepal to southest Tibet	2400-3400
R.vaccinioides	Bush	East Nepal to Yunnan	2400-3400
R.virgatum	Bush	East Nepal to southest Tibet; not common in Nepal	2100-2900
R.wallichii	Shrub	East Nepal, Sikkim, Bhuta,	3000-4200
R. wightii	Shrub	East Nepal to southeast Tibet	3500-4300

(Source: Press *et al.*, 2000, Milleville, 2002, Shrestha *et al.*, 2012))

(Note: Highlited species were considered for present (first part) study)

2.3 Rhododendron species under study

Following descriptions of various *Rhododendron spp.* are based on Rajbhandari & Watson, 2005.

2.3.1 *Rhododendron anthopogon* D. Don

This is commonly known as bearded Rhododendron and it is known by different names by different ethnic and group's and language viz. Kemba khoru by Sherpa, Palu by Tibetans, Sunpaati by local people and Dhupi gurans in Nepali. The specific name of this species is derived from Greek anthos = flower, pogon = beard, which literally means bearing bearded flowers, alluding to the corolla throat which is hairy.This is one of the earliest Himalayan species to be described by D. Don from specimens sent by Dr. N. Wallich form Gossainthan in Nepal in 1821.The heady odour of this species emits incites headache and nausea at higher altitudes, yet the delicate pink flowers that it produces is evidently one of the daintiest in the genus (Pradhan *et al.,* 1990).

Morphological characteristics:

Small erect, strongly aromatic evergreen shrub, 15-80(-100) cm tall; branchlets clothed with short- and long – stalked lacerate brown scales, scales later becoming bristles; leaf- bud scales deciduous. Leaves aromatic, ovate, elliptic, orbicular, oblong –elliptic or oblong, 1-3(-4) x 0.5 -2 cm, apex rounded or obtuse, shortly mucronate, base rounded or obtuse, dark green and sparsely scaly above, densely (usually pale or brown) scaly beneath with closely overlapping, lacerate scales, petioles 2-5 mm, scaly. Flowers 4-8(-10) in terminal dense subcapitate racemes; pedicels 2-4 mm, scaly. Calyx conspicuous, deeply 5- lobed, 2-5 mm, lobes, lanceolate or elliptic, scaly outside, margin densely ciliate. Corolla salver- shaped (narrowly tubular with spreading lobes), 1.2 – 1.9 cm long, ca. 2 cm across, white, creamy, pale yellow, pale pink, deep rose or reddish, glabrous (not scaly) outside, tube densely white pilose within; lobes five, rounded, often translucent. Stamens usually 5-9, unequal, 2-5 mm long, inclined within corolla- tube; filaments glabrous. Ovary ovoid or conic, 5(rarely 4) -celled, scaly.Style short, straight, not scaly. Capsuled ovoid or conic, 2-4(-5) mm, scaly (Rajbhandari *et al*., 2005)

Distribution:*R.anthopogon* is reported to be distributed from 3300 – 5100 m in Nepal (Press at al., 2000). In Sagarmatha National Park (SNP), it is found between Dingboche and

Lobuche and along Namche-Everest Base Camp (EBC) route but less common in this area but common in Gokyo valley (Shrestha et al., 2012). This is widely distributed species from Kashmir eastward through Nepal, Sikkim, Bhutan, Arunachal Pardesh and South –East Tibet (Rajbhandari & Watson., 2005)

Ecology: It grows commonly in open habitats and dwarf scrubland on or usually above the treeline, often forming dense stands and associated with *R.setosum;* open pastures, alpine ridges; rocky hillsides, rhododendron forest margins, and dry peaty soil; between 3000-4800 m (Rajbhandari & Watson., 2005).

Flowering: Mid-May to July

Photochemicalconstituents: Leaves gives ursoleic acid and quercetin. Leaves produce aromatic oil containing pinene, d-limonene, 3-cyclohexane,linalyl propanoate, butanoic acid, caryophyllene, copaene, caryophyllene oxide,guaiol, selinene,2- nephthalenemethanol and naphthalenamine (Gabbriella *et al*., 2010).

Uses: The leaves are highly aromatic and often used (with Junipers) as one of the major sources of incense in the monasteries (Rajbhandari & Watson, 2005). Decoction of leaves is used in cold, cough, and chronic bronchitis. It is widely used as incense for its aromatic properties. Furthermore, its leaves and fresh flowers are used as tea by Himalayan healers to promote digestive system, stimulate appetite and relieve liver disorders. *R. anthopogon* is also used for sore throat, common cold and lung problems. The essential oil, Anthopogon oil, as it is usually referred to in Nepal, is obtained by steam distillation of the aerial parts of *R. anthopogon.* Also known as Sunpati oil, this oil is a good natural source of sweet herbal, a faintly balsamic essence. *R. anthopogon* essential oil can be used on the skin and hair. According to Himalayan aromatherapy, this oil stimulates the nervous system and has been used for treating sore muscles and gouty rheumatic conditions (Pradhan *et al*., 1990).

Taxonomic notes: although the corolla colour is quite consistent within the populations, it is very variable across the geographical range of the species from white, cream, pale yellow, pale pink, pink to deep rose or reddish. Some authors recognize the white flowered plants (especially those in cultivation) as var. album Davidian, but this has little merit in wild populations (Rajbhandari & Watson., 2005).

2.3.2 *Rhododendron arboreum* sm.

This is commonly known as scarlet abrorescent rhododendron and laligurans in Nepali. It is the national flower of Nepal since 1962.It is tallest rhododendron tree in Nepal is probably measuring 18 m and located at Mangalbar, 1850 m, around 10 km west-north-west of Ilam. It grows mainly in the forests and is oftenseen growing together with oak between 2300 and 2700 m. Its beautiful pure forests are nearly always between 2700 and 3000 m and mainly in the eastern half of the country. It has a preferences for slopes and hill ridges. Its trunk can be gnarled on its smallest trees. Its strong rootsgenerally run on the ground. The bark can appear to be cracked when old. The *R.arboreum* is the only rhododendron in Nepal on which orchids grow (Northeast Nepal)(Milleville, 2002).

Variation and sub species:

The red *R.arboreum* is the dominant one of the species. The pink is called *R.arboreum roseum* and white one *R.arboreum album* (from the Latin 'albus', meaning white). Among the subspecies are the *R.arboreum s. sp cinnamomeum* (from the Greek 'kinamon = cinnamon), which has an indumentum of a rusty or cinnamon colour that can be scratched with the nail and *R.arboreum s. sp.campbelliae* (after Dr. Campbell, a long time British resident of Darjeeling in the nineteenth century), with a brown and wooly indumentum on the underside of the leaf (Milleville, 2002).

Morphological characteristics:

Shrub 1-2 m or tree up to 16(-18) m, evergreen; young shoots puberlous to tomentose. Leaves lanceolate, oblong- lanceolate or elliptic – oblanceolate, 6.5- 1.7 x 2- 5 cm, apex acute or shortly acuminate, base cuneate; upper surface glossy dark green, veins deeply impressed, glabrous; lower surface densely matted, 1- layered, white, silvery or fawn tomentose, (sometimes obscured byloose red- brown tomentum) or loosely whitish or fawn floccose; petioles 8-15 mm, tomentose. Racemes compact, 10 – 20(-25)- flowered; pedicels 5- 10 mm, hairy, glandular. Calyx 1- 2 mm, shallowly 5 –lobed, glandular or glabrous.Corolla tubular – campanulate, 3-4.5 cm long, 4- 5 cm across, 5- lobed, bright red, rarely pink or more rarely white, with darker spots and nectar pouches.Stamens 10, shorter than corolla.Ovary conic or oblong, white tomentose; style glabrous. Capsules oblong (rarely oblong- oval), slightly curved, 1- 2.5 cm, hairy (Rajhbhandari *et al.,* 2005).

Distribution:In SNP, it is found below Surke to Phortse and Pangboche (Shrestha *et al.,* 2012).Pakistan, North West India (Sikkim), Bhutan, South West China (S Xizang), North East India, North Myanmar (Rajbhadari & Watson,2005).

Taxonomic notes: This geographically widespread tree rhododendron is found over a wide range of altitudes and exhibits a complex pattern of different forms which are more or less distinct (Rajbhadari & Watson, 2005).

Flowering: (January-) February-May (-June)

Phytochemicalconstituents: It has different classes of secondary metabolites such as alkaloids, steroids, flavonoids, terpenoids, anthraquinones, phlobatanins, saponins, glycosides, tannins and reducing sugars. These secondary constituents vary in type in different parts of the plant. The presence of these secondary metabolites signifies the potential of *R. arboreum* as a source of therapeutic agent. Infrared spectroscopic analysis of the methanolic extract of flowers leaves, bark, stem and roots of *R.arboreum* indicated the presence of O-H, C=O, C-H, C=O, C=C, NH, NO_2 and C-O-C bond stretching. The medicinal values of *R.arboreum* are due to the presence of the detected metabolites (Nisar*et al.,* 2011).

Uses: Widely used for fuel, flowers are used as appetizer, bark juice used for cough,petals for menstrual disorders as well as chewed in case of fish bone stuck in the throat with a belief

that they dissolve fish bone and young leaves used for headache (Rajbhandari & Watson, 2005).

2.3.3 *Rhododendron barbatum* Wall ex G. Don

This is commonly known as bristly rhododendron and laal chimaal in local language. Its name derives from Latin *barbatus,* meaning bearded of provided with tufts of long weak hairs or bristles, which the young shoots and petioles of this species usually bears. The first specimen was discovered by Dr. N. Wallich from Gossainthan, Nepal and described by G. Don in 1834. Sir Joseph Hooker describes this in 1849 as "One of the most beautiful Himalayan species" (Pradhan *et al*., 1990).

Morphological characteristics:

Shrub or small tree to 2- 8 m, evergreen; trunk and branches with smooth reddish or grey – brown flaking bark; young shoots with stiff spreading bristles. Leaves elliptic, obovate or elliptic- oblanceolate, 7.5 – 17 (-23) x 2.5- 5 cm, apex acute, shortly acuminate or alomost mucronate, base, cuneate or narrowly rounded; upper surface glossy dark green, glabrous, veins deeply impressed; lower surface with a few dendroid hairs (especially when young) and stalked glands, usually almost glabrous; petioles 1- 2 cm, with characteristics long stiff spreading bristles 6- 10 mm, rarely glabrous or puberulous. Inflorescence 10 -20 flowered compact rounded racemose umbel, 10- 13 across; pedicels 0.5- 2.5 cm, glabrous (rarely floccose), eglandular. Calyx conspicuous, crimson, scarlet, pink or green flushed red, with 5 deep rounded lobes 5- 10 mm long, rarely shorter. Corolla tubular- campanulate, 5 –lobed, 2.8- 4 cm long, fleshy, crimson or scarlet (occasionally white flowers have been reprted), 5 nector pouches at the base. Stamens 10, 1-1.2.5 cm; filaments white, glabrous, rarely puberulous at the base; anthers purple black.Ovary conic, glabrous or short glandular hairy; style glabrous. Capsules oblong, slightly curved, 1.5 – 2 cm, bristly (Rajbhandari *et al.*, 2005).

Phytochemical constituents: There are nine compounds viz n-triacontane,hen-triacontol, friedelin,(β)-amyrin,olieanolicacid,(β) Sicosterol,butolonicacid,sitosterol,glucoside and 5,6,7,4-tetramethoxyflavone from the roots of R.barbatum (Rawat *et al.*, 2011).

Distribution: It is found Near Phunki Tenga,Theso(between Namche Bazaar & Thamo) & between Khumjung and Mong.North West India, Nepal, North India (Sikkim), Bhutan, North East India, South West China (S Xizang) (Rajbhandari & Watson, 2005).

Ecology: It grows in to high elevation forest of Abies, Picea, Tsuga dumosa and Rhododendron, often forming dominant stands in central Nepal; between (2400-)2700-3600 m (Rajbhadari & Watson, 2005).

Flowering :(February-) April- June

Uses:It contains andromedotoxin-a substance that helps reduce blood pressure (Joshi & Sharma, 2009).Dried leaves snuffed to cure sinusitis; acts as fish cattle poison (Rajbhandari & Watson., 2005).

2.3.4 *Rhododendron campanulatum***D. Don**

This is commony known as bell flowered rhododendron and Nilo chimaal in local language.*Rhododendron campanulatum* is very common and variable species which was discovered by Dr. N. Wallich and later discovered by D. Don in 1821. The first specimen recorded came from Gossainthan, Nepal. It was introduced into cultivation around the middle of Nineteenth century and has since been an important parent of several modern Rhododendron hybrids (Pradhan *et al*., 1990).

Morphological characteristics:

Large, widely branched, spreading evergreen shrub 1- 4 m or small tree to 6 m tall; branchlets glabrous. Leaves elliptic, obovate, oblong or oblong-ovate, sometimes elliptic – obovate, (7-) 9.5-14 x 3-7 cm, apex acute or subacute, base rounded or narrowly cordate, blade thinly coriaceous and flexible, margins weakly or not reflexed when dry; upper surface smooth (veins obscure), glabrous, shiny green, without metallic bloom; lower surface covered with a thin, suede- or velvet- like fawn, brown or rusty- brown continuous indumentum of capitellate (clustered sessile) hairs soft to the touch; petioles glabrous, 0.5 – 2 cm. Racemes lax, 6- 15-flowered, umbellate; pedicles 1- 2.5 cm, glabrous,. Calyx 5- lobed, minute, 0.5-1.5 mm, glabrous. Corolla broadly campanulate, 2.5 – 4.5 cm long, ca. 4 cm across, 5- lobed, white, bluish – purple, pale rose or lilac, with reddish spots within above and large blotch at base, dark pink, nearly purple in bud, fading to pale pink or white when old. Stamens 10, 1-4

cm, shorter than corolla; filaments white pr pale pink, glabrous or with few hairs at base; anthers purple- black.Ovary slender, oblong or sometimes conic, glabrous; style glabrous. Capsules slender or elongate- cylindric, slightly curved, 1.5 -2.5 cm (Rajbhandari & Watson, 2005)

Taxonomic note: This species is very similar to *R.wallichi* and some populations in Nepal appear to integrade suggesting hybridization. On this bases some authors treat *R.wallichi* as variety of *R.campanulatum.* Further field study on wild populations is necessary to resolve the taxonomy (Rajbhandari & Watson, 2005).

Distribution: In SNP, it is distributed from Namche to Pangboche along Namche-Everest Base camp route, and Namche to Dole along Namche- Gokyo route (Shrestha *et al.,* 2012). Rigionalyly, it is distributed in Pakistan, North West India, Nepal, North India (Sikkim), Bhutan, North East India, South West China (S Xizang) (Rajbhandari *et al.,* 2005)

Flowering: April-May- June

PhytochemicalConstituents: It contains terpenoids, steroids in the diethyl ether and chloroform extracts, tannins in the diethyl ether and methanol extracts, and glycosides, flavonoids, and saponins in the methanol extract. The diethyl ether and methanol extracts exhibited dose-dependent antimicrobial activity against *Staphylococcus aureus* and *Streptococcus faecalis* while no activity was observed against Gram-negative bacteria. These results suggest that *R. campanulatum* is a potential source of antibacterial agents (Paudel *et al.,* 2011).

Uses: Leaves are mixed with tobacco to make medicinal snuff useful in colds and hemicranias, chronic rheumatism, syphilis, and sciatic. Dried twigs and woods are used in phthisis and chronic fevers (Shrestha, 2009).

Ecology: It is common in forests , thickets, amongst rocks in Juniper scrub, on cliff's, more rarely in alpine zone, forming pure forests in mid elevations and mixed forest with *R campylocarpum* at higher elevations; between 2800- 4000(-4400) m (Rajbhandari *et al*., 2005).

2.3.5 *Rhododendron campylocarpum*D. Don

This is commonly known as curved – fruit rhododendron and Bango – phale gurans in Nepali and Pahenlo Chimaal by local.The specific name of *R. campylocarpum* is derived from Greek kampulos (campylos) = bent and karpos (carpus)= fruit refering to the arc- like fruits it carries.It is charming species with sulphur yellow flowers borne on rounded bushes covered with bright green foliage.The flowers are honey- scented while the glandular hairs on the leaf – stalk, calyx and capsules emits a resinous odour. The characteristics curved fruits and much smaller calyx clothed with glandular hairs distinguished this from*R. thomsonii* when not in bloom (Pradhan *et al.*, 1990).

Morphological characteristics:

Shrub to 1- 4 m or small tree to 6 m, evergreen; branchlets thinly covered with stalked glands. Leaves elliptic or ovate –elliptic, 3.2- 9 x 1.5 – 5 cm, coriaceous, apex rounded or apiculate, base shallowly cordate; upper surface green, slightly shiny, midrib impressed, veins obscure; lower surface whitish green, glabrous; petioles 0.5 – 2 cm, with sparse glandular hairs. Racemes terminal, lax, 3- 7(-13) - flowered, umbellate; pedicels, with sparse long glandular hairs. Calyx small, 1.5 – 3(-5) mm long, glandular, lobes 5, rounded. Corolla campanulate, 2.5- 4.5 cm long, 3- 4 cm across, 5 lobed, pale yellow to bright yellow, sometimes with a red basal blotch. Stamens 10, unequal, 1.2- 2.5 cm. Ovary conic- cylindric, 5- 7 mm, densely covered with stalked glands; style ca. 2 cm, glamdular at base, glabrous above. Capsules 1.3- 3 cm, strongly curved (Rajbhandari & Watson, 2005).

Taxonomic notes: The Nepalese plants belong to the typical subspecies. *Rhododendron campylocarpum* subspecies *caloxanthum* (Balf. f. & Farrer) D.F. Chamb., that is restricted to China (East Xizang, North West Yunnan) and North West Myanmar, and distinguished by its glabrous filaments and smaller, suborbicular leaves (4-6 x 3.5- 4 cm) (Rajbhandari & Watson, 2005).

Distribution: In SNP, it is distributed between Namche and Deboche along Namche-Everest Base Camp route and between Namche and Dole along Namche-Gokyo route.Between Khunde and Kyangjuma,Tyangjuma and deboche, and Phortse Tenga and Dole (Shrestha et al., 2012).Regionally, it is distributed in Nepal, North India (Sikkim), Bhutan, North East India, South West China (South& South East, Xizang) (Rajbhandari &Watson, 2005).

Flowering: May-june

Ecology: It grows in high elevation forests and high scrubland with *Tsuga, Abies, Junipers,* and *Rhododendron* (especially with *R. wallichi* and *R .campanulatum*), stony slopes; between 3000-4000(-4400) m (Rajbhandari & Watson, 2005)

2.3.6*Rhododendron hodgsonii*Hook. f.

This is commonly known as hodgson's Rhododendron and Korlinga in local language.It is the most common Rhododendron in SNP. In summer the leaves are broad, and spreading all-round the plant while in winter rolled up, shriveled and pendulous from the tips of the branches. It is found at the buttom of the valleys, on the rocky spurs or slopes and ridges of the hills, in open places, or in gloomy pine (Pradhan *et al.*, 2005).

Morphological characteristics:

Shrub or tree, 3- 7(-12) m tall, evergreen; trunks and branches with smooth, pinkish- brown, flaking bark; branchlets densely white tomentose. Leaves oblong – obovate, obovate, oblong-elliptic, elliptic or oblong- lanceolate, 10-28(-40) x 5-12 cm, thickly coriaceous, apex rounded, rarely obtuse, base obtuse or rounded; upper surface dull or glossy dark green, veins not promonent; lower surface finely silver- white to pale- brown tomentose (soft to touch) with shortly fimbriate cup- shaped hairs; petioles 2- 5 cm, very stout, tomentose. Racemes compact, or sometimes somewhat lax, umbellate, 15-20 cm across, 12- 20- flowered; pedicels 1.5 – 4 cm, tomentose. Calyx minute, 1 mm, lobes 7-8, triangular or ovate, tomentose outside. Corolla tubular- campanulate, 3-5.8 cm long, 4- 5 cm acroos, 7-8 lobed, fleshy, crimson, purple, magenta, deep purple, purple, pinkish-red, rose, pink or deep lilac, usually with dark blotches at base. Stamens 14- 18, 1.4-3.4 cm, shorter than corolla; filaments pale pink, glabrous, rarely puberulous at base; anthers purple- black.Ovary oblong or slender, 9-12 celled, tomentose; style glabrous, pale pink, eglandular; stigama yellow- green. Capsules oblong or somewhat slender, 2.6- 6 cm, slightly curved, tomentose (Rajbhandari & Watson, 2005).

Distribution: Shady slopes near Tyangboche(Millevile, 2002).Nepal, North India(Sikkim), Bhutan, North East India, South West China (S Xizang) (Rajbhandari & Watson, 2005).

Ecology: It grows in high elevations forests with Tsuga, dumosa, Abies, Rhododendron and Betula (can form pure stands by itself), open forests and hillside shrubland, rocky ground above tree line between 2600- 3800 m (Rajbhadari & Watson, 2005).

Flowering: April-May

Uses: The wood is hard and used for making cups, spoons, and saddles (Shrestha, 2009).Yak butter and cheese is also packed in the glossy foliage for presentation to local market (Rajbhandari & Watson, 2005).

2.3.7*Rhododendron lepidotum*Wall ex G. Don

This is commonly known as scaly Rhododendron and bhaale sunpaati in local language. *R.lepidotum*consists of extremely variable groups of plants, which could as done earlier by Sir Joseph Hooker and later R. L. Stevenson in Species Rhododendron published by the Royal Horticultural Society, London in 1930, easily be taken for separate species (Pradhan *et al.*, 1990).

Morphological characteristics:

Erect or mat – forming aromatic resinous subshrub (5 -) 20-80(-150) cm tall, evergreen or sometimes semi- deciduous, new shoots often flushed red; branchlets scaly. Leaves obovate, oblanceolate or elliptic, (0.3-) 2.4 x (0.2-)0.4 -1 cm, usually coriaceous, apex acute or rounded, base cuneate or rounded; upper and lower surfaces green (pale yellow), conspicuously scaly, scales yellow- brown, not closely overlapping; petioles 1- 3 mm, scaly. Racemes terminal, umbellate, 1- 2(-4) - flowered; pedicels slender, 1.2- 2.5 cm, scaly.Calyx small, lobes 5, rounded, 1-4 mm long, scaly. Corolla rotate or campanulate, 0.8 -1.5 cm long, 2- 2.5 cm across, 5- lobed, red, pink, purple, white or yellow, often spotted, scaly outside. Stamens 8- 10, longer than corolla tube; filaments pubescent towards base. Ovary conic, 5-celled, scaly; style short, sharply bent, not scaly. Capsules oblong, conic or oblong- ovate, 4-9(-20) mm, scaly (Rajbhandari *et al.*, 2005).

Phytochemical Constituents: An unusual natural product named 2,4,6-trihydroxacetophenone-3, 5-di-C-beta-D-glucoside were isolated from aerial parts of *Rhododendron lepidotum* grows in Tibet, along with other 17 known compounds: hopenol-B, lupeol, ursolic acid, avicularin , quercetin, myricetin, hyperoside, myricetin-

3'-O-beta-D-xyloside, (+)-taxifolin-3-O-alpha-L-arabinopyranoside, (+)-taxifolin-3-O-beta-D-glucopyranoside,lyoniside,confluentin,2-(hydroxyphenyl) (Xhou *et al.*, 2012).

Distritribution: In SNP, it is found in handi khola to above thukla, chukkung and near the fourth lake of Gokyo (Shretha *et al.*, 2012).Regionally, is is distributed from Pakistan, North West India, Nepal, North India (Sikkim), Bhutan, North East India, South West China (West Sichuan, South Xizang, North WestYunnan),North Myanmar (Rajbhandari & Watson, 2005).

Ecology: Common in forests mixed with evergreen Oak, Pinus, Abies etc.,a very common component of high elevations dwarf shrubland with Junipers and other dwarf Rhododendron, on cliffs, among boulders, stony slopes and grasslands; between (2100-)2400-4700 m (Pradhan *et al.,* 2005)

Flowering: mid - May – July

Uses: Leaf decoction as insecticidal spray. Bark taken as tea is purgative. Leaf as aromatic stimulant (Shrestha, 2009)

2.3.8 *Rhododendron nivale*Hook. f.

This is commonly known as snow Rhododendron and Hiun Gurans in Nepali.No other flowering shrub in the world attains the altitude that *R. nivale* reaches, and is the most tenacious among flowering shrubs. Sir Joseph Hooker most vividly describes his unique find "The latest to bloom and the earliest to mature its seeds, by far the smallest in foliage, and proportionally largest in flower, the most lepidote in vesture, humble in stature, rigid in texture, deformed in habit,yet the most odoriferous, it may be recognized, even in the herbarium, as the production of loftiest elevation on the surface of the globe of the most excessive climate- of the joint influences of a scorching sun by day, and the keenest forest at night.. During genial weather, when the sun heats the soil to 150 degree, its perfumed foliage scents the air, while to snowstorm and forest it is insensible, blooming through all, expanding its little purple flowers to the day, and only closing them to wither after fertilization has taken place (Pradhan *et al.,* 1990)

Morphological characteristics: Small, much-branched prostrate or erect shrubs, usually forming dense cushions, (0.3)0.6-0.9(1.2) m tall; branches densely dark ferruginous scaly. Petiole 0.5-2(3) mm, scaly; leaf blade elliptic, ovate or rounded, 0.3-0.5(-1.2) cm; base

broadly cuneate; apex acute or rounded, occasionally mucronulate; abaxial surface yellowish green to fawn, scales contiguous or nearly contiguous, 2-colored, pale golden and dark brown intermixed, usually either equal in number or paler scales predominating; adaxial surface dark green, densely scaly, gray-white or golden. Inflorescence 12-(3) flowered. Pedicel 0.5-0.15 cm, scaly, occasionally pubescent; calyx lobes (0.1)2-4(4.5) mm, oblong or narrowly lanceolate, scaly, margin ciliate and/or scaly; corolla broadly funnelform, pink, or lilac to purple, (0.7)0.9-1.4(1.6) cm, tube (2.5)3- 4(6) mm, outer surface rarely scaly, often pubescent, throat pubescent; stamens (8)10, longer or shorter than corolla, filaments villous towards base; ovary 12 mm, scaly; style usually longer than stamens. Capsule ovoid to rounded, 35 mm, scaly (Flora of China, 2005).

Distribution: In SNP, it is distributed in Namche-Everest Base Camp route frequently above Lobuche. It distributed from Western Nepal, through Sikkim, Bhutan and South East Tibet at 4500- 6000 m.

Ecology: As it named, eight month of the year it is buried under many feet of snow, for remaining four it is frequently snowed and sunned in the same hour.

Flowering: June- July

Uses: Leaves used for incense (Shrestha, 2009)

2.3.9*Rhododendron setosum*D. Don

This is commonly known as bristly Rhododendron and Balu in local, Tsallu Gurans in Nepali. It was discovered by Buchanan-Hamilton in Nepal and described in 1821 by D .Don.It is a dwarf plant inhabiting the higher mountain slopes above 3000 m. Like all higher alpine plants, it is late to flower (June- July) and early to set seeds (October).This plant emits such intense aroma at the high altitudes on sunny day that discomfort and headache it causes is often unbearable (Pradhan *et al*., 1990).

Morphological characteristics:

Erect shrub (0.1-) 0.3-0.6(-1) m tall, evergreen, strongly aromatic, branches interwined; young shoots densely scaly, densely bristly (setose). Leaves ovate, obovate, oblong-obovate, oblong, elliptic or oblong- elliptic, 6- 17(-20) x 4- 7 mm, apex rounded, truncate or shallowly retuse, margin inrolled, bristly, base rounded; upper surface dark green,

conspicuously yellow – white sacly; lower surface pale green, red- brown scaly, apex and base of midrib bristly; petioles 1- 3 mm, scaly, bristly. Racemes dense, terminal, umbellate, 2-5- flowered; pedicels 3- 8 mm, scaly, pubescent. Calyx deeply 5- lobed, lobes ovate- elliptic, 4- 7 mm long, reddish or crimson- purple, scaly, sometimes ciliate. Corolla widely funnel- shaped, 1.3 – 2 cm long, 2 -2.5 cm across, 5- lobed, pale to deep purple or pink, glabrous outside. Stamens 8- 10, longer than corolla; filaments pubescent towards base. Ovary conic, 5- celled, pubescent, scaly; style straight, longer than stamens. Capsules conic or oblong- ovoid, 5- 6, scaly (Rajbhandari & Watson, 2005).

Distribution: In SNP,it is found from Syangboche to Lobuche as well as near to fourth Lake of Gokyo,between Dingboche and Chukkung, Pheriche valley of khumbu (Shrestha et al., 2012). It is distributed from Eastern Nepal, Sikkim, Bhutan and South East Tibet at 3000-5500 m. It is quite common in Sikkim (Rajbhandari & Watson., 2005).

Ecology: It is very common (especially in eastern areas) and gregarious in high elevation dwarf Rhododendron scrubland (especially with *R.anthopogon*), along open mountain ridges, open rocky areas and alpine pasture; between (3400-) 3800-4800(-5600) m (Rajbhandari & Watson, 2005).

Flowering: May to July

Uses: Dried leaves are burnt as incense in monasteries, for making aromatic oil, perfumery and cosmetics (Shrestha, 2009).

2.4The Family Ephedraceae

The family Ephedraceae, also known as Mormon- tea family contains the single genus *Ephedra* and placed in the class Gnetopsida and order Ephedrales have about 40 species of shrubs. *Ephedra L.* is a genus in the drier parts in arid regions of the subtropics and tropics (Ekbal *et al*., 2014). It is widely distributed through the North Temperate Zone and high mountains of South America (Stapf, 1889; Florin, 1933).

Due to extensive extinction in the geological past, modern Ephedraceae possess as a set of morphological characters disjunct from the other two monotypic families of the Gnetales (Gnetaceae and Welwitschiaceae) as well as from other living seed plants including Cycads, Ginkgo, Conifers and Angiosperms (Pearson, 1929; Cutler, 1939; Martens 1971; Fu *et al*. 1999).

2.4.1 Characteristics of genus Ephedra

Plants of this family have branches or twigs including nodes and internodes, the node is enlarged, and the internode is longitudinally furrowed. Leaves usually bear 2 (-4) parallel veins, and are opposite and decussate or ternately whorled; they are free and linear in early Cretaceous fossils and a few extant species, but fused into a sheath at a node in most living species. Female cones of extant Ephedra are compound, and have a few pairs or whorls of bracts, but only the uppermost pairs or whorl of bracts enclose 1-3 seeds. The bracts are fleshy, coriaceous, or membranous when the cone is mature. The characteristic female reproductive unit bears an outer envelope and an inner integument, the integument is elongated into a micropylar tube passing through the apical opening of the outer envelope. Male cones have many whorls or pairs of bracts, each bract usually subtends an axillary male reproductive unit and the male reproductive unit consists of a pair of bracteoles enclosing an inner antherophore. Each antherophore has 3-12 stalked or sessile bilocular synangia at the tip (Yang, 2014).

2.4.2 Distribution of *Ephedra gerardiana*

2.4.2.1 World distribution

Ephedra gerardiana is distributed in Afghanistan, China, North India, Nepal, Pakistan, Sikkim, Tajikistan (Fu *et al.*, 1999).

2.4.2.2 Distribution in Nepal

In Nepal, it occurs in the arid climate of the rain shadow areas of the Great Himalaya, where more than 300 mm of annual water deficit occurs. Soils are strongly calcareous owing to restricted leaching and to the preponderant occurrence of limestone parent materials. Cattle grazing that selectively removes palatable plants such as grasses appears to be one of the important factors promoting the *Ephedra gerardiana* community (Kojima, 1990).

2.4.3 Ecology of *Ephedra gerardiana*

Ephedra gerardiana is found in the Himalayan Mountains from Afghanistan to Bhutan. It prefers dry mountains and high mountain deserts. In Nepal, it is most often found growing near *Juniperusrecurva* and Rhododendron species. Animals also appear to enjoy the stimulating effects of the plants. *Ephedra gerardiana* may be grown from seed, and requires rocky soil. It needs very little water to survive, and even thrives in soil with high salt content (Ratsch, 1998).

2.4.4Characteristics of *Ephedra gerardiana*

Shrubs small, to 15 cm; woody stems buried in soil, parts above ground scabrous, rarely almost smooth; branchlets directed upward, dark green, later brown, short, stout, usually with 1-3 nodes, internodes 1-1.5 cm long, 1.5-2 mm wide, longitudinally furrowed, often scabridulous. Leaves opposite, 2-3 mm, connate for ca. 2/3 their length. Pollen cones solitary or 2 at nodes, small, and 2-5 mm long, ca. 2 mm wide, sessile; bracts in 3 or 4 pairs; staminal column exserted for ca. 1/2 its length, with 8 sessile anthers. Seed cones solitary, sessile or shortly pedunculate, subglobose at maturity, 5-7 mm in diameter; bracts in 2 or 3 pairs, connate for 1/4-1/3 their length, the apical pair for ca. 2/3 their length, red and fleshy at maturity; integument tube short, ca. 1 mm. Seeds 1 or 2, oblong or obovoid-oblong, 4-6 mm long, 1.5-3 mm wide, exserted, apex obtuse or subacuminate (Wen *et al*., 2006).

The common names of *Ephedra gerardiana* viz.Amsania, Budagur, Chefrat, Khanda Ma Houng (Tibetan), Ma-huang (Chinese), Narom (Pakistani), Oman (Pashto), Raci, Sang Kaba (Sherpa, 'kaba incense'), Sikkim Ephedra, Soma, Somalata (Sanskrit, 'moon plan' Nepali), Somalatha Plant, Thayon (Ladakhi), Tootagantha (Hindi), Tseh (Tamang), Uman (Pashto)

Plate 2.1*Ephedra gerardiana*

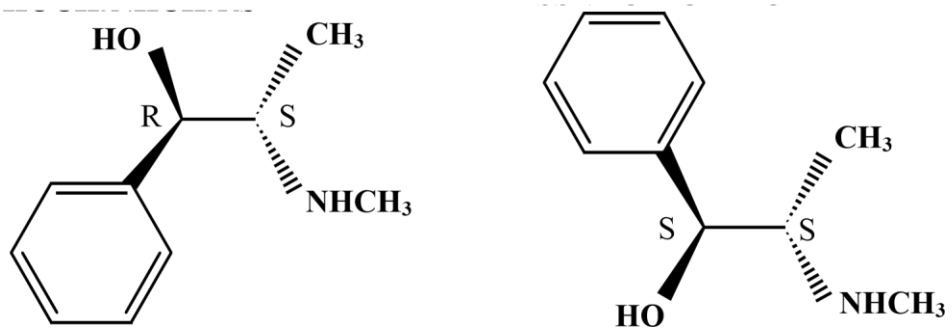
2.4.5Chemical constituents of *Ephedra* spp.

The major active ingredients of Ephedra are alkaloids that constitute 0.5 to 2.5 percent of the total mass, and are referred to as ephedrine-type alkaloids (Blumenthal and King, 1995). The six optically active alkaloids that have been isolated from Ephedra species are (-)-ephedrine, (+)-pseudoephedrine, (-)-N-methylephedrine, (+) N-methyl pseudoephedrine, (-)-norephedrine, (+)-norpseudoephedrine. Usually, (-) ephedrine is the major isomer that comprises 30 to 90 percent of total alkaloid fraction accompanied by (+)-pseudoephedrine, with trace amount of other ephedrinetype alkaloids (Blumenthal and King, 1995). The total content of ephedrine type alkaloids depends on the species of Ephedra, time of the year of harvest, weather conditions, and altitude where the plant grows (Blumenthal and King, 1995), and can exceed 2% (Bruneton, 1995). This variation according to environmental conditions explains why some Ephedra containing dietary supplements of the same brand often show

alkaloid content markedly differ at from label claims and also variation among lots when analyzed chemically (Gurley *et. al*., 2000).

Preparation of ephedrine-type alkaloids from crude plant material involves an acid/base extraction procedure (Reti, 1953). In addition to the extraction from plants, ephedrine-type alkaloids can also be chemically synthesized, and most of the ephedrine and pseudoephedrine used in western medicine has been manufactured synthetically. However, these synthetic ephedrine-type alkaloids differ from the natural forms in that they are racemic, i.e., optically inactive, because they are made up of two enantiomorphic isomers (Abourashed *et al.*, (2003).

In addition to the ephedrine-type alkaloids, other alkaloids and amino compounds have been isolated from different species of Ephedra such as the macrocyclic spermine alkaloids, Ephedradines A-D, kynurenic acid derivatives, cyclopolyglycine, methanoproline amino acids, flavones, flavanols, tannins, carboxylic acids, volatile terpenes (Schaneberg *et al.*,(2003).



(-)-ephedrine (+)-pseudoephedrine

Figure 2.1 Chemical structures of (-)-ephedrine and (+)-pseudoephedrine (adapted from Abourashed *et al.*,(2003)

2.4.6 Pharmacology

Ephedrine is a sympathomimetic substance that stimulates both central nervous system and adrenergic receptors (Abourashed *et al.,* 2003). Stimulation of 1-adrenergic receptors produces contraction of vascular smooth muscle, increased contractile force of the heart and arrhythmias, glycogenolysis, gluconeogenesis, hyperpolarization, and relaxation of intestinal smooth muscle. Stimulation of 2-adrenergic receptors decreases insulin secretion, platelet aggregation and the release of norepinephrine from the nerve terminals, and causes contraction of vascular smooth muscle. Stimulation of 1-adrenergic receptors increases force and rate of contraction of the heart, increased velocity of conduction through the atrioventricular node, and increased rennin secretion. When used in therapeutic doses, stimulation of 2-adrenergic receptors causes relaxation of the smooth muscle of the blood vessels and bronchi (Mack, 1997). Stimulation of -adrenergic receptors including 3-subtype involve in lypolysis and non-shivering thermogenesis (Abourashed *et al.,* 2003). In addition to its direct effects, ephedrine also displays indirect sympathetic activation releasing norepinephrine from sympathetic neurons (Abourashed *et al.,* 2003). Ephedrine also has central nervous system (CNS) stimulant effects similar to those of amphetamines, but less pronounced (McEvoy, 2000).

2.4.7 Uses of Ephedra

Various Ephedra spp. have been used since time for the treatment of various human ailments.

2.4.7.1 Uses in western medicine

In Western medicine, ephedrine is used for the treatment of nasal congestion due to hay fever, allergic rhinitis, asthma, and common cold (WHO, 1999). Also, ephedrine salts are prescribed in the form of nasal sprays to relieve congestion and swelling. When injected subcutaneously, ephedrine prevents hypotension during anesthesia. Orally, it has been used in treating certain forms of epilepsy, nocturnal enuresis, myasthenia gravis, and urticaria accompanying angioneurotic edema.Pseudoephedrine, taken orally, is an effective nasal decongestant (Morton, 1977).

2.4.7.2 Uses in Chinese medicine

The Ephedra species have been used in traditional Chinese medicines (TCM) for at least 5,000 years (Morton, 1977). In traditional Chinese medicines, dried stems of Ephedra species are used to alleviate symptoms caused by common cold, influenza, asthma, bronchitis, nasal congestion and hay fever. They were also used for the treatment of arthritis, fever, hives, lack of perspiration, headache, aching of joints and bones, wheezing, and low blood pressure (Leung and Foster, 1996). The tissue used in TCM is the dried green stem of one of three Ephedra species (*Ephedra sinica, E. equisetina and E. intermedia*), which are usually boiled in water and administered as a hot tea. In contrast to the diaphoretic uses properties of ma-huang (stem part), the root and rhizome of Ephedra species, called mahuanggen, have antiperspirant property and are employed to treat spontaneous and night sweating (Leung, 1990).

2.4.7.3 Ethnobotanical uses in Nepal

In Nepal, dried *E.gerardiana* bundles are burned as incense during cremation ceremonies. The smoke is pleasant and spicy, and has been compared to the smell of a forest life. The ashes which are left behind may also be taken as snuff. However, the plant is only taken internally by powerful shamans and high lamas, and is treated with much respected and reverence (Ratsch, 1998). Dry *E. gerardiana* stalks are boiled in water for ten minutes to make a stimulating tea.

2.4.7.4As dietary supplements

Approximately a decade ago, a new usese of Ephedra different from traditional directions had been widespread in the United States (Fukushima K., 2004). Focusing on the thermogenic and lipolytic effects of Ephedra, dietary supplements containing Ephedra extracts have been commercially promoted and used as a mean of weight reduction and energy enhancement (Josefson, 1995). On the contrary, there has been increasing number of reports of adverse reactions associated with the use of Ephedra containing products submittedinto the U.S. Food and Drug Administration (FDA). Food and Drug Administration,1994 reported reactions varied from the milder adverse effects such as nervousness, dizziness, tremor, headache, and gastrointestinal distress to chest pain, myocardial infarction, hepatitis, stroke and death. Of the 140 reports submitted to the FDA between June 1997 and March 1999, 47% involved

cardiovascular symptoms and 18% neurological symptoms. Severe hypertension was the single most frequent adverse effect followed by tachycardia, myocardial infarction, stroke, seizure. Ten events resulted in death and 13 produced permanent impairment (Haller and Benowitz, 2000).

2.5 Molecular marker tools in biodiversity research

2.5.1 Plant Molecular Systematics

Biological systematics is referred as the theory and practice of grouping individuals into species, arranging those species into larger groups and assigning names to those so as to produce classification (Judd *et al.,* 1999). In the history of taxonomy four main approaches have been recognized *viz.* a) artificial, b) natural and phyletic, c) phenetic and d) cladistics or phylogenetic (Stuessy, 1990).

2.5.1.1Molecular genetic markers

Molecular markers are not normal genes with biological effects but are similar to constant landmarks in the genome. Markers and genes lie close to each other on same chromosome and stay together in each generation of plants. The relative position of markers in a chromosome and their closeness to any specific genes can be used to create a linkage map (Semagn *et al.*, 2006). Such maps can be used to study associations between economically important traits or Quantitative trait loci (QTL). Besides, such markers can also be linked to qualitative traits and can also be employed for the assessment of genetic diversity, phylogeny and diagnostic development (Shrestha *et al.*, 2003; Mondini *et al.*, 2009; Arif *et al.*,2010;Shrestha *et al.*, 2010Lübberstedt and Varshney, 2013).

An ideal molecular marker should have following desirable traits (Agarwal *et al.*, 2008; Jonah *et al.*, 2011):

1. It should be polymorphic and evenly distributed throughout the genome
2. It should provide adequate resolution of genetic differences
3. It should generate multiple, independent and reliable markers
4. It should be simple, quick, and inexpensive to generate.
5. It should need small amounts of tissues
6. It should have linkage to distinct phenotypes

7. It should require no prior information about the genome of an organism
8. It should be codominantly inherited in order to distinguish homozygosity and heterozygosity
9. It should have easy exchange of data between laboratories

It is difficult to find a single marker possessing all the properties mentioned above, Hence, the selection should be determined by type of study undertaken.

Genetic markers can be divided into three categories: i) non-PCR-based techniques or hybridization based techniques, ii) PCR-based techniques and iii) DNA sequencing based techniques.

i) Hybridization based markers / Non-PCR based techniques

-Restriction Fragment Length Polymorphism (RFLP)

-Variable Number of Tandem Repeats/Minisatellites/Microsatellites

ii) PCR-based techniques

PCR with Arbitary primers

-Random Amplified Polymorphic DNA (RAPD)

-Arbitarily Primed Polymerase Chain Reaction (AP-PCR)

-DNA Amplification Fingerprinting (DAF)

-Amplified Fragment Length Polymorphism (AFLP)

-Inter-Simple Sequence Repeats (ISSR)

Specific Primed PCR (Site-Targeted PCR)

-Sequence Characterized Amplified Region (SCAR)

-Cleaved Amplified Polymorphic Sequence (CAPS)

-Microsatellites/Simple Sequence Repeats (SSR)/ Short Tandem Repeats (STRs), Sequence Tagged Microsatellite (STMs) or Simple Sequence Length Polymorphism (SSLP)

iii) DNA sequencing based markers

-Nuclear rDNA sequences such as Internal Transcribed Spacer (ITS)

-cpDNA and mtDNA sequences

-Single Nucleotide Polymorphisms (SNP)

PCR based techniques

Some widely used PCR – based markers are reviewed in following section.

2.5.1.2Inter Simple Sequence Repeat – PCR (ISSR-PCR)

The basic premise is that SSR loci are dispersed evenly throughout the genome and the chances of 'hitting' two SSRs with a common motif, oriented on opposing DNA strands, within an amplifiable distance of one another is high enough that single primer amplifications should yield a high degree of polymorphic bands (Condit and Hubbell, 1991).

Inter simple sequence repeat (ISSR) technique is a PCR based technique which involves amplification of DNA segment present at an amplifiable distance in between the two identical microsatellite repeat regions oriented in opposite direction (Zietkiewicz *et al.*, 1994). The technique uses microsatellites, usually 16-25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. The microsatellite repeats can be di-, tri-, tetra-, or penta-nucleotides (Reddy *et al.*, 2002). The primers can be either unanchored (Meyer *et al.*, 1993; Gupta *et al.*, 1994; Wu *et al.*, 1994) or anchored at 3' or 5' end with 1 to 4 degenerate bases extended into flanking sequences (Zietkiewicz *et al.*, 1994) as shown in Figure 2.2.

ISSR combines the benefit of RAPD and microsatellite analysis with the universality of these. ISSR has high reproducibility possibly due to the use of longer primers (16-25 mers) as compared to RAPD primers (10 mers) thus allowing use of high annealing temperature (45-60^{o}C) leading to higher stringency and the primers aren't proprietary (as in SSR-PCR). It also has a distinct advantage of simplicity, quickness and efficiency and there is no use of radioactivity at all. The amplified products are 200 – 2000 bp long and can be detected by agarose and polyacrylamide gel electrophoresis (Reddy *et al.*, 2002). ISSRs are inherited as dominant markers following simple Mendelian inheritance (Gupta *et al.*, 1994; Tsumura *et al.*, 1996; Ratnaparkhe *et al.*, 1998;Wang *et al.*, 1998). In some cases, however, they are also inherited as co-dominant markers which can be used as marker to distinguish between homozygotes and heterozygotes (Wu *et al.*, 1994; Akagi *et al.*, 1996; Wang *et al.*, 1998;Sankar and Moore, 2001).

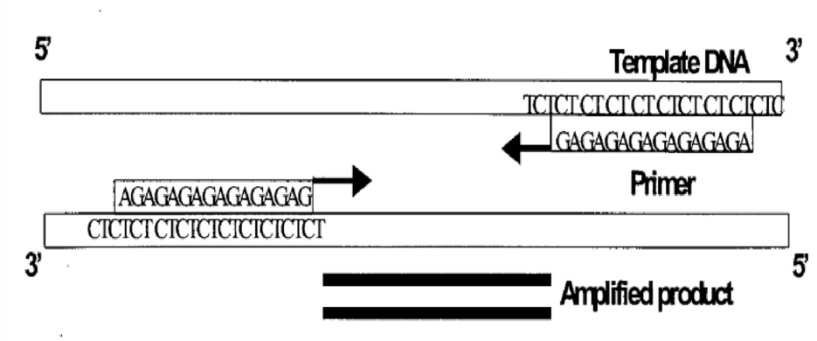


Figure 2.2 Schematic representation of ISSR-PCR of an unanchored single primer $(AG)_8$ (Source: Reddy *et al.*, 2002)

ISSRs are used for varied purposes one of them being estimation of genetic diversity at inter- and intra-specific level such as in crop species like rice (Joshi *et al.*, 2000), wheat (Nagaoka and Ogihara, 1997) etc. Similarly it has been used in the study of genetic structure of different medicinal plants like *Dendrobium* sp. (Yang *et al.*, 2010), *Swertia* spp. (Joshi and Dhawan, 2007; Zhang *et al.*, 2007; Tamhankar *et al.*, 2009), *Podophyllum hexandrum*(Alam *et al.*, 2009; Naik *et al.*, 2010), *Rheum* spp. (Wang *et al.*, 2012a; Wang *et al.*, 2012b) etc.

2.5.1.3Random Amplified Polymorphic DNA (RAPD)

RAPD is anarbitrarily primed PCR-based marker system. In this system, the total genomic DNA of an individual is amplified by PCR using a single, short (usually about ten nucleotides/ bases) and random primer. The primer which binds to many different loci is used to amplify random sequences from a complex DNA template that is complementary to it (maybe including a limited number of mismatches). Amplification can take place during the PCR, if two hybridization sites are similar to one another and 3000bp apartb and in opposite directions (Kumar *et al*., 2011).The amplified fragments generated by PCR depend on the length and size of both the primer and the target genome. The PCR products (up to 3 kb) are separated by agarose gel electrophoresis and imaged ethidium bromide (EB) staining. Polymorphisms resulted from mutations or rearrangements either at or between the primer-binding sites are visible in the electrophoresis as the presence or absence of a particular RAPD band (Xu, 2010).

RAPD predominantly is a dominant marker system. This system yields high levels of polymorphism and is simple and easy to be conducted. First, neither DNA probes nor sequence information is required for the design of specific primers. Second, the procedure does not involve blotting or hybridization steps, and thus it is a quick, simple and efficient

technique (Farooq *et al.*, 2002).Third, relatively small amounts of DNA (about 10 ng per reaction) are required and the procedure can be automated, and higher levels of polymorphism also can be detected compared with RFLP. Fourth, no primer development is required, and the primers are nonspecies specific and can be universal. Fifth, the RAPD products of interest can be cloned, sequenced and then converted into or used to develop other types of PCR-based markers, such as sequence characterized amplified region (SCAR), single nucleotide polymorphism (SNP), etc. However, RAPD also has some limitations/disadvantages, such as low reproducibility and incapability to detect allelic differences in heterozygotes (Jing, 2010).

2.5.1.4Simple Sequence Repeats (SSR) marker

SSRs, also called microsatellites, short tandem repeats (STRs) or sequence-tagged microsatellite sites (STMS), are specific primed PCR-based markers. They are randomly tandem repeats of short nucleotide motifs (2-6 bp/nucleotides). Di-, tri- and tetra-nucleotide repeats, e.g. (GT)n, (AAT)n and (GATA)n, are widely distributed throughout the genomes of plants and animals (Wang *et al.*, 2009). The copy number of these repeats varies among individuals and is a source of polymorphism in plants. Because the DNA sequences flanking microsatellite regions are usually conserved, primers specific for these regions are designed for use in the PCR reaction. One of the most important attributes of microsatellite loci is their high level of allelic variation, thus making them valuable genetic markers. The unique sequences bordering the SSR motifs provide templates for specific primers to amplify the SSR alleles via PCR. SSR loci are individually amplified by PCR using pairs of oligonucleotide primers specific to unique DNA sequences flanking the SSR sequence. The PCR-amplified products can be separated in high-resolution electrophoresis systems (e.g. Agarose Gel Electrophoresis (AGE) and Polyacrylamide Agarose Gel Electrophoresis (PAGE)), capillary gel electrophoresis and the bands can be visually recorded by fluorescent labeling or silver-staining (Song *et al.*, 2010).

SSR markers are characterized by their hyper-variability, reproducibility, co-dominant nature, locus-specificity, and random genome-wide distribution in most cases. The advantages of SSR markers include that they can be readily analyzed by PCR and easily detected by PAGE or AGE. SSR markers can be multiplexed, have high throughput genotyping and can be automated. SSR assays require only very small DNA samples (~100 ng per individual) and low start-up costs for manual assay methods (Rajeev *et al.*, 2005).

A comparison between different markers frequently used in various studies is enlisted in Table 2.2 below.

Table 2.2 Comparison of five widely used DNA markers in plants (Semagn *et al.*, 2006)

Markers / **Features**	**RFLP**	**Microsatellite**	**RAPD**	**AFLP**	**ISSR**
Genomic Abundance	High	Medium	Very high	Very high	Medium
Part of genome surveyed	Low copy coding regions	Whole genome	Whole genome	Whole genome	Whole genome
Amount of DNA required	High	Low	Low	Medium	Low
Type of polymorphism	Single base changes, insertion, deletion	Changes of length of repeats	Single base changes, insertion, deletion	Single base changes, insertion, deletion	Single base changes, insertion deletion
Level of polymorphism	Medium	High	High	Very high	High
Effective multiplex ratio	Low	Medium	Medium	High	Medium
Marker index	Low	Medium	Medium	High	Medium
Inheritance	Codominant	Codominant	Dominant	Dominant	Domina nt
Detection of alleles	Yes	Yes	No	No	No
Ease of use	Labour intensive	Easy	Easy	Difficult initially	Easy
Automation	Low	High	Medium	Medium	Medium
Reproducibility	High	High	Intermediate	High	Medium to high

Types of probes/primers	Low copy genomic DNA or cDNA clones	Specific repeat DNA sequences	Usually 10 bp random nucleotides	Specific sequences	
Cloning and/or sequencing	Yes	Yes	No	No	No
Radioactive detection	Usually yes	No	No	Yes/no	No
Development/start -up costs	High	High	Low	Medium	Medium
Utility for genetic mapping	Species specific	Species specific	Cross specific	Cross specific	Cross specific
Proprietary rights status	No	No (some are licensed)	Licensed	Licensed	No

2.5.2DNA sequencing based barcode markers and their application

In the present generation, DNA sequencing has become very popular for genetic level studies. It implies the direct approach in studying the polymorphism at DNA level by determining the nucleotide sequences of a defined region. Two basic strategies of DNA sequencing were devised in the mid-1970s *viz.* a) Chemical degradation based method (Maxam and Gilbert, 1977) and b) dideoxy chain termination Enzyme based method (Sanger's *et al.,* 1977). Of these two, the Sanger method is the most widely used. In this method, DNA is synthesized from four deoxy nucleotide triphosphates. Each new nucleotide is added to the 3′-OH group of the last nucleotide added (Sanger *et al.,* 1977). The dideoxy method gets its name from the critical role played by synthetic nucleotides that lack the -OH group at the 3′ carbon atom. A dideoxynucleotide can be added to the growing DNA strand but when it is added chain elongation stops because there is no 3′ OH for the next nucleotide to be attached to. For this reason, the dideoxy method is also called the chain termination method.

DNA sequencing provides highly robust, reproducible and informative datasets and can be applied to different levels of discriminatory potential by choosing appropriate genomic target regions (Chhipi Shrestha, 2012). On the negative side, DNA sequencing can be prohibitively tedious and expensive when very large numbers of individuals have to be assayed such as in population genetics, phylogeography and marker assisted plant breeding programs (Weising *et al.,* 2005). Various sequencing based markers such as nuclear ribosomal DNA (rDNA) Internal Transcribed Spacer (ITS), chloroplast DNA markers (*rbcL, matK, trnH-psbA*) and mitochondrial (COX1) are widely applied in the phylogenetic reconstruction and diagnostic development for DNA based studies (Shrestha *et al.,* 2003).

Most recently, with the introduction of Genome Analyzer in 2005, has revolutionized sequencing chemistry from first generation to next generation sequencing. The efficiency of the labelling turned for 84 kilobases (kb) per run to 1 gigabase (GB) per run.

The different characteristics and properties of barcode markers are summarized in Table 2.3.

Table 2.3 Characteristics of barcode markers (Hollingsworth *et al.*, 2011)

Marker	**Genomic source**	**Type**	**Amplicon length range (bases)**	**Frequency of amplicons with mononucleotide repeats ≥ 10 bases**
nrITS	Nuclear	Transcribed spacers and 5.8S gene	407 – 1630	0.013
matK	Plastid	Protein coding	862 – 910	0.235
rbcL	Plastid	Protein coding	654 – 654	0.000
trnH-psbA	Plastid	Intergenic spacer	226 – 934	0.296

2.5.3Chloroplast DNA (CpDNA)

The chloroplast DNA (cpDNA) of higher plants is a double stranded, circular molecule, ranging in size from 120 to 160 kb (Odintsova and Yurina, 2006). Typical chloroplast genome consists of large and small single copy regions (LSC, SSC respectively) separated by two duplicated inverted repeat regions (IRA and IRB) (Ravi, 2008). Among angiosperms, this genome is highly conserved in size, structure and gene content (Olmstead and Palmer, 1994).

Chloroplast DNA (cpDNA) has been used extensively to investigate phylogenetic relationship at a wide range of taxonomic levels in plants. Chloroplast genes are now used routinely to infer phylogenies because direct sequencing of polymerase chain reaction (PCR) produces makes it relatively easy to obtain sequence data. DNA sequences from several chloroplast genes, such as *rbcL*, *matk, ndhF, atpB, trnH-psbA,* and *rps,* have been used to estimate phylogenetic relationships at higher taxonomic levels. For example, *rbcL* has been widely used at family level or above (Price & Palmer, 1993).

It is widely recognized that molecular phylogenetic studies should include multiple markers to assure that the gene tree are an accurate representation of the species phylogeny (Doyle, 1992). The most widely used markers in plants are from chloroplast genes and the internal

transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA). Most cpDNA coding regions do not evolve rapidly enough to resolve relationship at these taxonomic levels (Doebley & al. 1990, Gaut & al., 1992). Several non- coding regions of cpDNA have potentially utility at lower taxonomic levels (Bohle *et al.*, 1994).

2.5.3.1Maturase K (*matK*)

Maturase K (*matK*) (formerly called ORFK) is one of the most rapidly evolving and shows a high level of species discrimination (Fazekas *et al.*, 2008; Lahaye *et al.*, 2008). It has length of about 1550bp and encodes the enzyme maturase kinase which is involved in splicing of type-II introns from RNA transcripts (Neuhaus and Link, 1987). Since *matK* is embedded in the group II intron of the gene trnK, it can be easily PCR-amplified with a primer set designed from the conserved regions of the genes *trnK*, rps16 and psbA (Neuhaus and Link, 1987). *MatK* has been widely used as a marker to construct plant phylogenies because of its rapid evolution and the ubiquitous presence in plants (Hilu and Liang, 1997; Kelchmer, 2000). However, failure of PCR amplification of *matK* in some taxonomic groups are also reported (Wolfe *et al.*, 1987). To circumvent this problem the primer set 390F and 1326R has been developed which amplifies around 930bp between postions 429 and 1313 of the *matK* genes and has worked in major angiosperms (Cuenoud *et al.*, 2002). Phylogenetically, the rate of evolution of *matK* was found suitable for resolving intergeneric as well as interspecies relationships in many angiosperms (Soltis *et al.*, 1998). The CBOL- Plant Working Group (CBOL Plant Working Group, 2009) tested *matK* in nearly 550 plant species and found that nearly 90% angiosperms samples were easily amplified and sequenced using single primer pair, though the success was limited in gymnosperms (83%) and much worse in cryptogams (10%). As more datasets are published, we can more accurately estimate the extent of primer universality for *matK*. Using the best currently available ‘universal’ primer pair (3F/1R; K. J. Kim) on diverse sample sets typically resulted in *matK* PCR and sequencing success of ca. 70% in angiosperms. Use of a secondary primer pair (390F/ 1326R; can increase amplification and sequencing success by another ca. 10%. This *matK* recovery rate clearly needs improvement for plant barcoding to be cost-effective and efficient. Furthermore, *matK* is not recoverable from some bryophyte and fern groups with available primer sets, most of which were designed for angiosperms. Ferns in particular represent a challenge for *matK* recovery as genome rearrangements mean that the gene is not flanked by conserved *trnK* exons in some clades, creating additional difficulties in generating full-length *matK* sequences from which to design primers for the barcode region.

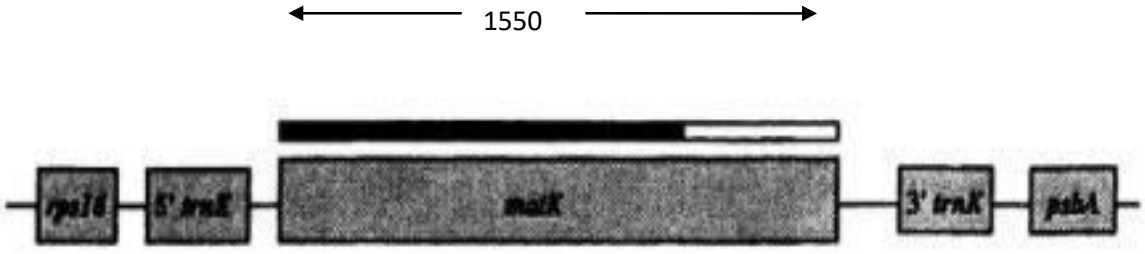


Figure 2.3 Structure of *matK* region. Black portion of bar above *matK* gene which is approximately 1,000bp region that is typically sequenced. (Soltis *et al.,* 1998).

2.5.3.2Ribulose-1,5-bisphosphate carboxylase-Large Subunit (*rbcL*)gene

The *rbcL* gene is located in the large single-copy region of the chloroplast genome and encodes the large subunit of ribulose 1,5-bisphosphate carboxylase /oxygenase (RUBISCO) whereas the small subunit of RUBISCO is encoded by rbcS, a nuclear gene (Soltis *et al.,* 1998). RUBISCO is a photosynthetic enzyme and was the first gene that was sequenced from the plants (Newmaster *et al.,* 2006). It has a high amplification success rate in a broad range of flowering plant, gymnosperm, and cryptogam species, plus high sequence quality among seven loci tested (CBOL Plant Working Group, 2009). Most of the phylogenetic studies suggest that *rbcL* is best suited to reconstruct the relationships down to the generic levels, but is not useful for species level (Soltis *et al.,* 1998). Furthermore, in order to obtain enough species discrimination, entire 1430 bp needs to be sequenced, which acts as a limiting factor for its use as a barcoding sequence because an ideal DNA barcoding region should be short enough to amplify from degraded DNA and analyzed via single-pass sequencing (Chase, 2007). The solution for this was to amplify short sequences with enough variability. Primers for PCR amplification and sequencing for such short sequence within the *rbcL* gene have been developed accordingly for most of the taxa. The primer for *rbcL* (i.e., *rbcL*a-f and *rbcL*a-r) amplifies 550-600bp from 5' region in all major taxonomic groups (Kress and Erickson, 2007).

The *rbcL* region has a high amplification success rate in a broad range of flowering plant, gymnosperm, and cryptogam species, plus high sequence quality among seven loci tested (CBOL Plant Working Group, 2009). The *rbcL* region showed the lowest divergence (0.83%) among 11 potential barcoding loci tested for the differentiation of two species in Solanaceae, namely *Atropa belladonna* L. and *Nicotiana tabacum* L. (Kress *et al.,* 2005). The barcode

region of rbc*L* is also easy to sequence, and align in most land plants and provides a useful backbone to the barcode dataset, despite it having only modest discriminatory power.

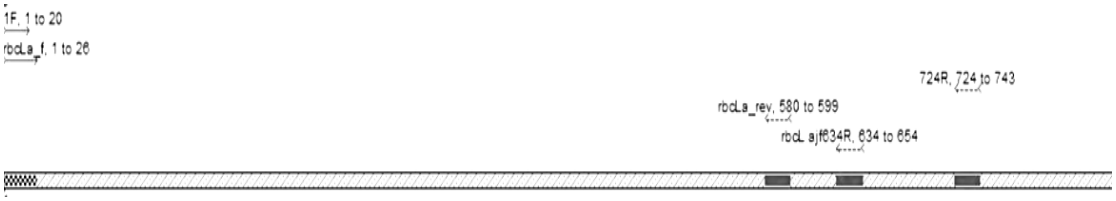


Figure 2.4 Location of binding of different primers in *rbcL* region of *Arabidopsis thaliana*.

2.5.3.3*trnH-psbA* intergenic spacer

The size of the *trnH–psbA* region of most flowering plants ranges between 340 and 660 bp. This region shows the highest amplification success rate (100%) and discrimination rate (83%) among nine loci tested, including ITS, *rbcL*, and *matK* (Kress *et al.*, 2005; Kress & Erickson, 2007). Therefore, this intergenic spacer appears to be a useful region for the differentiation of various species including medicinal plants from their adulterants.This region is straightforward to amplify across land plants, and is one of the more variable intergenic spacers in plants. Because of the high amplification success rate and species discriminatory power exhibited by this small segment of DNA, Kress *et al.,* (2005) proposed it along with nrITS for DNA barcoding in plants. Along with these desirable properties there are some obstacles. In some angiosperms, *trnH-psbA* has an exceedingly short (300bp) length which do not have enough sequence variation for species discrimination of those taxa (Shindel and Miller, 2005). Along with this, due to presence of rps19 gene, pseudogene and poly A/T structure within *trnH-psbA* locus in some monocot and conifer species it may go up to 1000 bp, which can lead to problems in obtaining bidirectional sequences (Chase, 2007; Hollingsworth, 2009; Zhu *et al.,* 2010). To overcome such problems, many researches have proposed the use of *trnH-psbA* with combination of *matK* (Chase, 2007; Erickson *et al.*, 2008). The trnH–psbA region has been used to distinguish the medicinal species *Artemisia annua* L. (Asteraceae) and *A. capillaries* Thunb. from their adulterants derived from *A. carvifolia* Buch. -Ham. Ex Roxb.using Kimura 2-parameter analysis (Liu and Ji, 2009). The trnH–psbA region was efficient in distinguishing various *Dendrobium* species, with relatively high interspecific variation (ranging from 0.3% to 2.3%) and intraspecific variation (ranging from 0% to 0.1%)(Yao *et al.,* 2009). It has been used successfully in a range of barcoding studies and is an obvious choice of a supplementary barcode. In directly comparable sample sets it has higher species discrimination success than rbcL+matK in groups such as *Ficus* and

Alnus and improved resolution in complex groups such as *Quercus* and *Salix*. The presence of duplicated loci can lead to problems in a small number of groups (e.g. *Pinus; cycads; Eryngium*). In some conifers and monocots, the region is in exceeds 1000 bp, whereas in bryophytes it can be less than 100 bp.

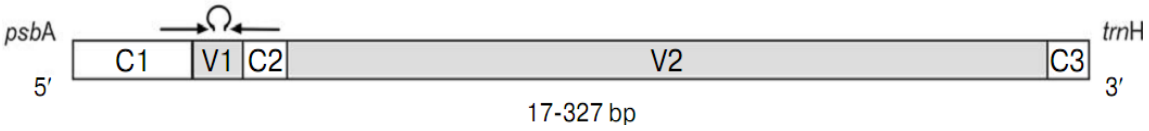


Figure 2.5 General scheme of organization of the *trnH-psbA* intergenic spacer. Also shown are conserved (C1, C2, C3) and variable (V1, V2) regions (Degtjareva *et al.*, 2012)

2.5.3.4Internal Transcribed Spacer (ITS)

The ITS region comprises the ITS1 intergenic spacer, *5.8S rDNA*, and the ITS2 intergenic spacer (ITS1–*5.8S*–ITS2), with size ranging from 400 to more than 1000bp in total, which lies between 18S small subunit and 26S large subunit genes, with size ranging from 400 to greater than 1000bp and has been used for the phylogenetic studies, species discrimination, barcoding and authentication of herbal plants due to the technical ease of amplification and sequencing (Alvarez and Wendel, 2003; Kress *et al.*, 2005). The ITS1 and ITS2 are the part of nuclear ribosomal DNA (rDNA) transcript but are not incorporated in the ribosomes and play a role in the maturation of nuclear rRNAs, bringing the large and small subunits into close proximity within a processing domain (Baldwin *et al.*, 1995). This function suggests that ITS1 and ITS2 are under some evolutionary constraint in structure and sequence (Hershkovitz and Zimmer, 1996).

The internal transcribed spacers from nuclear ribosomal DNA (nrITS) are an obvious choice of a supplementary barcode in groups in which direct sequencing is possible. The greater discriminatory power of nrITS over plastid regions at low taxonomic levels is well established in plant molecular systematics, and it has been clear from the outset that in groups where nrITS works well, it will be frequently used as a DNA barcode (Hollingsworth *et al.* 2011).

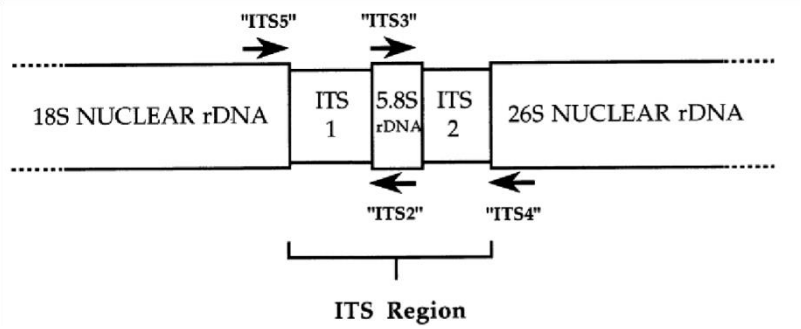


Figure 2.6 Organization of ITS region. Arrrows indicate orientation and approximate position of primer sites. Primer names (in quotation marks). (Baldwin *et al.*, 1995)

The advantage of using nuclear DNA in plant systematic over chloroplast DNA is reviewed by Riesenberg (1997) as firstly, certain nuclear sequences evolve more rapidly than any cpDNA sequences, which allow a much refined level of discrimination at the population level, and secondly, a nuclear gene represents both maternal and paternal genealogies whereas cpDNA represents only maternal genealogy (Hollingsworth *et al*. 2011). The limitations of the internal-transcribed spacers have been well documented in general terms. However, without a formal empirical estimate of the number of plant groups in which the problems are likely to occur, it is not possible to know whether these are truly pervasive problems that are likely to impact a large proportion of barcoding studies, or if these problems will affect a relatively small number of species/ samples relative to the gain in discriminatory power (Hollingsworth *et al*. 2011).

2.6.4 Cladistic based phylogenetic approach

Cladistic or phylogenetic approach is the latest and most widely accepted approach at present. Cladistic can be difined as the concepts and methods for the determination of branching patterns of evolution (Stuessy, 1990). According to Henning (1979), in phylogenetic systematics, various characteristics of plants are referred to as 'characters' and new charaters are called 'derived characters' (apomorphies). A group composed of an ancestor and all of its descendants is known as monophyletic group, which is recognized on the basis of shared derived characters (or synapomorphies). Synapomorphies arise in ancestral groups and are present in all of its members.

2.6.5 Molecular Phylogeny & methods of its construction

Phylogeny refers to the evolutionary or the topology of the tree of life and evolution refers not only to descent with modification but also involves the process of separation of lineages (Judd *et al.*, 1999). Phylogenetic assessment can be carried out from extreme micro to macro evolutionary levels (Avise, 1994). Previously, while describing organismal relationships, various approaches rely on the data obtained from comparative morphology, physiology and other assayable phenotypic features (Shrestha, 2001). The reasons for employing molecular genetic markers in studying molecular systematic as mentioned by Avise (1994) are: (a) Molecular data are genetic because phylogeny is the stream of heredity, only genetically transmitted characters are informative for phylogenetic estimation. Organisms are influenced by environment and are specially pronounced in plants and such environmentally induced variation can be misleading in the absence of molecular evidence; (b) Molecular methods can be applied to any living creatures from microbes to whales; (c) Molecular techniques can access an unlimited pool of genetic variability; (d) Molecular data can distinguish homology from analogy; (e) Molecular data provide common standards for measurement of divergence (i.e., inter-specific, intra- specific, inter-generic etc); (f) Molecular approaches facilitate mechanistic appraisals of evolution. Modern molecular methods such as DNA sequencing provide previously inaccessible information with regards to fundamental molecular basis of evolutionary change. (g) Molecular approaches are challenging and exciting. Newly developed PCR and DNA sequencing based methods have provided great opportunities in the reconstruction of phylogeny.

The tree consists of nodes (taxonomic units) and branches (pathway connecting nodes) which summarize the evolutionary relationships among organisms. In the tree, species or

higher taxa represent the Operational Taxonomic Units (OTUs), the external nodes represent extant OTUs and internal nodes represent the ancestral units. Branch lengths show the number of evolutionary changes along each ancestral- descendant pathway. Phylogenetic tree can be constructed using two main approaches *viz.* 1) distance based approaches and 2) character state based approaches (Avise, 1994). UPGMA cluster analysis method (Sneath and Sokal, 1973), Neighbour-joining method (Saitou and Nei, 1987), Distance Wagner Method (Farris, 1972) are distance based methods whereas Wagner parsimony (Farris, 1970), Dollo parsimony (Farris, 1977) and generalized parsimony (Swofford and Olsen, 1990) are character based methods.

A cladogram depicts the evolutionary relationship by constructing a phylogenetic tree and is rooted using an out-group or groups (Judd *et al.,* 1999). The cladogram is constructed such that the number of changes from one character to the next is minimized and the principle behind is the rule of parsimony which means that any hypothesis that requires fewer assumptions is a more defensible hypothesis (Lipscomb, 1998). In other words, parsimony analyses the number of characters that change in parallel or reverse (homoplasy) (Judd *et al.,* 1999). The simplest, and most common, measure of homoplasy is the consistency index (CI), which equals the minimum amount of possible evolutionary change (the number of genetic switches) divided by the actual tree length (the number of actual genetic changes on the tree) (Judd *et al.,* 1999). CI is designed to vary between 0 and 1. Zero for a character that changes many times in the tree and 1.0 for a character that changes only one & it is represented as:

$$CI=\frac{\text{total character state chages expected given the data set}}{\text{actual number of steps on the tree}}\times 100$$ (Judd *et al.,* 1999)

CI has various disadvantages as uninformative charaters are omitted from the study and also sensitive to the number of taxa in an analysis (Sanderson and Donoghue, 1989). To circumvent these limitations of CI, Forey *et al.,* (1992) designed the Retention Index (RI). The RI is computed by calculation of the maximum possible tree length, which is the length that would occur if the derived character state originated independently in every taxon in which it appears (i.e., if all taxa with the derived character state were unrelated). The RI equals the maximum length minus the actual length, divided by the maximum length minus the minimum length & it is represented as:

$$RI=\frac{\text{maximum length}-\text{actual length}}{\text{maximum length}-\text{minimum length}}\times 100$$ (Forey *et al*., 1992)

2.6.5.1 Model based Phylogeny

All phylogenetic methods make assumptions, whether explicit or implicit, about the process of DNA substitution (Felsenstein, 1988). The strategies for model selection include Akaike Information Criterion (AIC) (Akaike, 1974), Bayesian Information Criterion (BIC) (Schwarz, 1974) and performance-based Decision Theory (DT) (Minin *et al.,* 2003).

In this study the BIC has been used for generating model based phylogeny. The BIC (Schwarz, 1974) provides an approximate solution to the natural log of the Bayes factor. It is represented as:

BIC = -2 ln Li + ki ln n

Where ki is the number of parameters in model i, ln Li is the Maximum Likelihood (ML) score (i.e., with all parameters fixed to their ML point estimates), and n is the sample size. The smaller the BIC, better the model fits the data. The various models tested in this study are represented in the Table 2.4.

Table 2.4 Different type of nucleotide substitution models with their base frequencies and substitution rates

Model	Base frequencies	Substitution Rates	References
GTR	unequal	AC≠ AG≠ AT≠ CG≠ CT≠ GT	Tavaré, 1986
HKY	unequal	AC=AT=CG=GT≠ AG=CT	Hasegawa *et al.*,1985
JC	equal	AC=AG=AT=CG=CT=GT	Jukes and Cantor1969
K80	equal	AC=AT=CG=GT≠ AG=CT	Kimura, 1980
SYM	equal	AC≠ AG≠ AT≠ CG≠ CT≠ GT	Zharkikh, 1994
F81	equal	AC=AG=AT=CG=CT=GT	Felsenstein, 1981

Where, GTR= General Time Reversible, HKY= Hasegawa, Kishino, and Yano, JC= Jukes and Cantor, SYM=Symmetrical Model, K80 = Kimura 1980, F81 = Felsenstein 1981

2.7DNA barcoding technology

Identification of plant species is critical in varied areas such as plant ecology, environment, food, agriculture, health etc. Plants are generally identified using morphological characteristics which requires special training in plant taxonomy, on-hand identification tools (like taxonomic keep books, flora information, scientific papers etc.) and most importantly

suitable plant materials (herbarium specimen) preferably with the reproductive structures. In the absence of reproductive structures it is almost impossible for many species to be correctly identified.Moreover, this morphological – based approach to the task of routine species identification has four significant limitations. First, both phenotypic plasticity and genetic variability in the characters employed for species recognition can lead to the incorrect identifications. Second, this approach overlooks morphologically cryptic taxa, which are common in many groups (Knowlton 1993; Jarman & Elliott 2000). Third, since morphological keys are often effective only for a particular life stage or gender, many individuals cannot be identified. Finally, although modern interactive versions represent a major advance, the use of keys often demands such a high level of expertise that misdiagnoses are common.Therefore, the limitations inherent in morphology- based identification system and the dwindling pool of taxonomists signal the need for a new approach. TheDNA barcoding technology to remedy the limitations of morphological identification systems for taxon recognition (Hebert *et al.,* 2003a; Hebert & Gregory, 2005).

DNA barcoding is an emerging tool in molecular biology for rapid species recognition and identification based on short DNA sequences (Hebert *et al*., 2003). The core idea of DNA barcoding is based on the fact that short pieces of DNA (barcode) can be found that vary only to a very minor degree within species but with much greater variation between species (www.barcodinglife.org). DNA barcoding is becoming an increasingly popular means of diagnosing species, based on the principle that sequence divergence is ordinarily much lower among individuals of a species than between closely related species. The approach could provide tools for the recognition of species limits and diversity, as an improvement on, or supplement to, traditional morphological taxonomy (Hebert *et al.,* 2003; Hebert & Gregory, 2005; Packer *et al.,* 2009). The barcode sequences provide unique identifier for a species, compared with barcodes of items in a supermarket. Such sequences are stored in a database such that samples can be identified by comparing sample sequences against the known sequences in the database such as NCBI, EMBL or DDBJ. This system of identification allows people without expert taxonomic knowledge to identify plant materials and especially from materials that may not be flowering, or may be seeds or seedlings, plant fragments in foods or medicines or even from gut of animals, timber samples etc. All the barcode data generated during barcode studies are deposited in a database called Barcode of Life Data System or BOLD.

In animals, the mitochondrial gene cytochrome c oxidase subunit1 (COI) has been established as an effective DNA barcode in several animal groups (Hebert et al., 2003a, 2004; Hogg & Hebert, 2004; Barret & Hebert, 2005). It is a favorable region for most animal species including some fungal species belonging to groups Ascomycota, Basidiomycota and Chytridiomycota (Chen *et al.*, 2010). However, *CO1* gene in plants aren't useful for identifying them due to the low amounts of variation in the genes as well as due to the variable mitochondrial genome (Chase *et al.*, 2005; Kress *et al.*, 2005; Chase *et al.*, 2007; Pennisi, 2007; Fazekas *et al.*, 2008). Thus it is important to screen either single or multiple regions in nuclear and plastid genomes in plants to identify appropriate DNA barcodes as shown by the studies depicted in Figure 2.10.

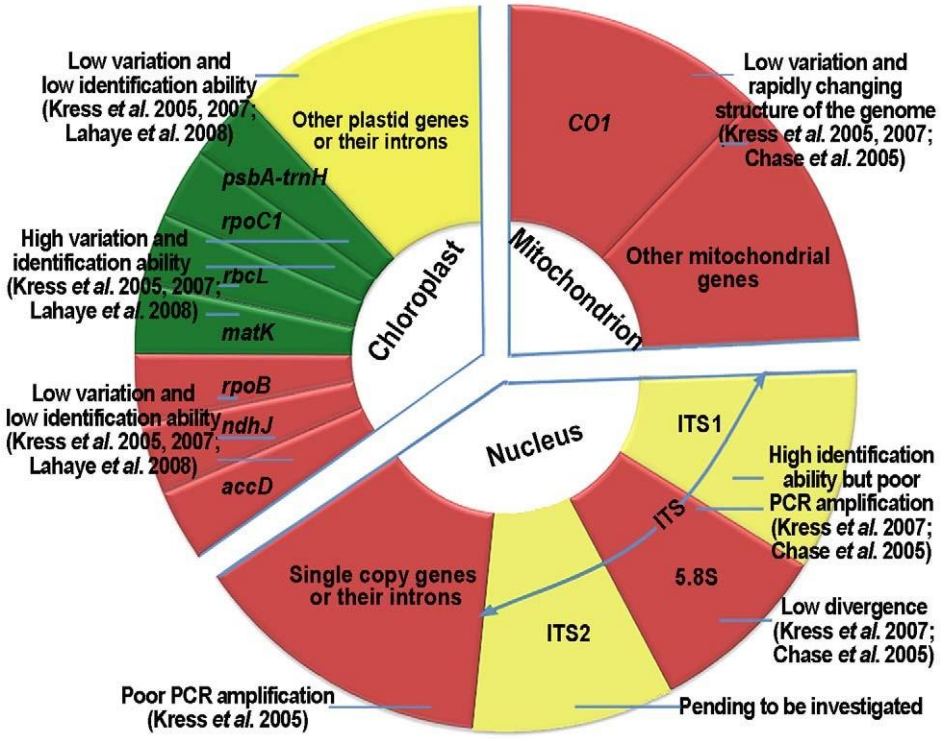


Figure 2.7 Genes from three genomes in plants that can be potential barcodes (Chen *et al.*, 2010)

In plants, although many studies have demonstrated that DNA barcoding is effective for species identification (Newmaster & Ragupathy, 2009b; Moniz & Kaczmarska, 2010), a standard DNA barcode for plant barcoding similar to *CO1* being used in animals remains elusive (Kress et al., 2005). Previous plant barcoding studies may be classified into four categories: (i) those looking for universal and consistent makers for large-scale land plants (e.g. Chase*etal*.,2005;Kress*etal*.,2005;Cowan*etal*., 2006; Newmaster *et al.*, 2006; Presting,

2006; Kress & Erickson, 2007; Sass *et al*., 2007; Chase et *al.,* 2007 Erickson *et al.,* 2008; Fazekas *et al.,* 2008; Lahaye *et al*., 2008a, 2008b; Devey *et al*., 2009; Ford *et al*., 2009; Chen *et al.,* 2010); (ii) those testing the identification power of different markers in a single family or genus (e.g. Hymenophyllaceae: Nitta, 2008; *Compsoneura* Warb.: Newmaster *et al.,* 2007; *Heracleum* L.: Logacheva *et al.,* 2008; *Aspalathus* L.: Edwards et al., 2008; *Acacia* Mill.: Newmaster &Ragupathy, 2009b; *Carex*L.:Starretal.,2009; *Crocus* L.: Seberg & Petersen, 2009; *Alnus* Mill.: Ren *et al*., 2010); (iii) those assessing the analysis methods and sampling strategies adopted by DNA barcoding studies (Little & Stevenson, 2007; Erickson *et al.,* 2008; Bergmann *et al.,* 2009); and (iv) those using one or several candidate markers in practical applications, such as distinguishing invasive from non-invasive species (Van de Wiel *et al.,* 2009) or identifying poisonous plants (Bruni *et al.,* 2010), species of economic importance (Newmaster & Ragupathy, 2009a), or medicinal species (Midgley&Turnbull,2003;He*etal*.,2010).

An appropriate DNA region or combination of DNA regions used in plant barcoding should be routinely amplifiable with universal primers, easily sequenced via single-pass sequencing, appropriate in length (300–800 bp; Kress *et al.,* 2005), variable enough to separate closely related species, and exhibiting less variability within species (Chase *et al.,* 2005; Cowan et al., 2006; Newmaster *et al*., 2007) . A number of candidate regions have been investigated. The nuclear ITS region and the plastid *trnH–psbA* intergenic spacer have been proposed as potential DNA barcoding regions for flowering plants (Kress *et al.,* 2005). The plastid gene *rbcL* has been proposed as a core DNA barcoding gene to discriminate plant species at the genus level in a tiered approach wherein a highly variable locus can be implemented if necessary (Newmaster *et al*., 2006). Chase *et al.* (2007) indicated that low levels of variation in plastid DNA made three regions necessary and proposed two combinations, namely *rpoC1*, *rpoB*, and *matK*, and *rpoC1*, *matK*, and *psbA–trnH*, as usable markers for land plants. Kress & Erickson (2007) proposed the combination of *trnH–psbA* and *rbcL* as a two-locus global DNA barcode for land plants. Newmaster *et al.* (2007) tested seven chloroplast loci for barcoding in *Compsoneura* and two regions (*matK* and *trnH–psbA*) showed promise as plant barcodes in nutmeg. Sass et al. (2007) tested eight DNA regions to discriminate cycads (Cycadaceae) and found that the ITScontains enough variability to identify most samples to the species level and is promising as a barcoding region. Fazekas et al. (2008) used eight chloroplast (cp) DNA regions (*rbcL*, *matK*, *rpoC1*, *rpoB*, *23S* rDNA, *trnH–psbA*, *atpF–atpH*, and *psbK–psbI*) and the mitochondrial *CO1* gene to identify species and found that the

resolution of a single locus ranged from 7% (*23S* rDNA) to 59% (*trnH–psbA*). Three chloroplast DNA regions (*rbcL*, *trnSGG*, and *trnH–psbA*) were tested in Hymenophyllaceae and the results showed that *trnH– psbA* had the greatest possibility using as a marker for DNA-based identification (Nitta, 2008). The Consortium for the Barcode of Life (CBOL) Plant Working Group compared the performance of seven candidate plastid DNA regions and recommended the combinationof*rbcL*and*matK* asthecorebarcodeforlandplants (CBOL, 2009). Chen *et al.*, 2010 proposed that ITS2 could serve as a novel universal barcode for plant identification(Morejon´ *et al.*, 2010). A study performed in *Alnus* (Betulaceae) showed *trnH-psbA* and ITS to be the best combination (Ren *et al.*, 2010). The chronological timeline of use of different barcode markers is depicted in Figure 2.8.

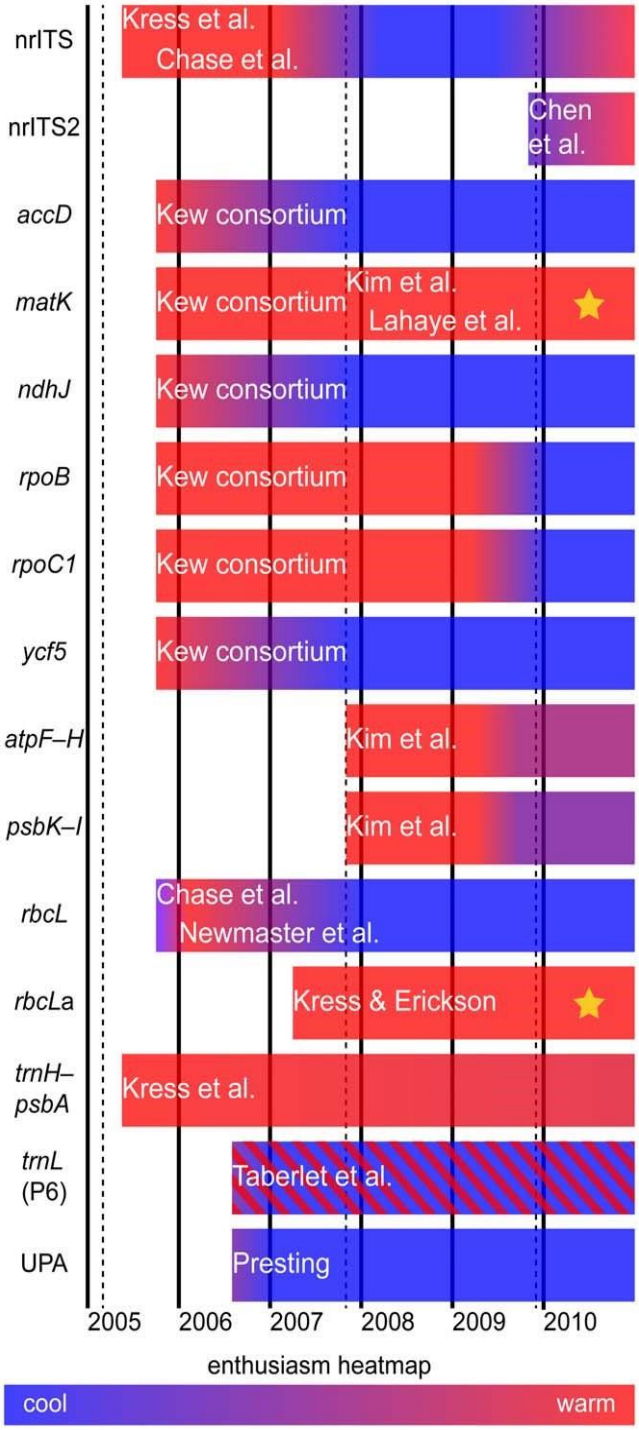


Figure 2.8 A schematic timeline of different markers used as plant barcodes. (Source: (Hollingsworth *et al.*, 2011)

2.6.1 Application of DNA Barcoding technology

DNA barcoding is a tool to identify species using a short and standardized DNA region (Hebert *et al*. 2004, Li *et al*. 2011a), and has been widely used in species identification and the discovery of new species (Newmaster *et al*. 2008, Hollingsworth 2011, Liu *et al*. 2011).

The DNA barcode is a short DNA sequence from a standard part of genome used for the identification of the species. The concept of applying DNA barcoding for the identification of global species was first proposed by Hebert *et al.,* (2003) and has gained significant momentum since then. To standardize the international use of DNA barcodes, the scientific community has made considerable efforts searching for suitable DNA regions for barcoding of every species (Kress *et al.,* 2005; Kress and Erickson, 2007; Min and Hickey, 2007; CBOL Plant Working Group, 2009). According to Li et al. (2011), DNA barcode should meet the following criteria: (i) high universality so that it can be sequenced routinely across plant species, (ii) high sequence quality and coverage that is amenable to the production of bidirectional sequences with minimum ambiguous base pairs, (iii) high discriminatory power to enable most species to be distinguished.

In animals, the partial region of mitochondrial cytrochrome C oxidase 1 (CO1) has been designated as standard barcode. It is, 650 base pair long and has been used for barcoding in birds (Kerr *et al.,* 2009), fish (Zemlak *et al.,* 2009), and insects (Bertsch, 2009). Because of its insufficient variations and low species discrimination power (Cho *et al.,* 1998, Cho *et al.,* 2004) and prone to rapid change in mitochondrial genome in plants (Adams and Palme, 2003), there was a need of some other alternative barcodes. Many findings revealed that nuclear region of the Internal Transcribed Spacer (ITS), chloroplast region of the *trnH-psbA* intergenic spacer, chloroplast coding regions like large subunit of ribulose-bisphosphate carboxylase (*rbcL*) and maturase K (*matK*) genes have been found suitable DNA barcodes for the loci tested in terms of high universality, quality and coverage of sequence, discriminatory ability (Kress *et al.,* 2005; CBOL Plant Working Group, 2009). The application of DNA barcoding in various field has been reviwed in following sections.

2.6.1.1 Species Identification and assigning specimens to known species

The original application of DNA barcoding was species identification (Hebert *et al.*2003) plans for barcoding include making a database of all COI barcoding sequences so that future specimens may be identified in case of animals. In case of plants, DNA barcoding, provides insights into species-level taxonomy in groups that have received inadequate taxonomic attention and to adequately characterize the diversity (Hollingsworth *et al.,* 2011). Along with this, barcoding could also be used as a quality control system to ensure that study specimens are identified correctly (Mitchell, 2008). It has been also used in enhancing the understanding of species limits in seed plants (Hollingsworth *et al.,* 2011). One problem with barcoding is that differentiating between species is only possible if arbitrary rules are employed.

Researchers must assume that intraspecific variation is significantly less than interspecific variation within the barcoding sequence region (Meyer and Paulay 2005; Langhoff *et al.,* 2009). One proposed method to differentiate between species is to set a standard threshold for the percentage of variation that is tolerated for specimen identification. Setting such a boundary is not a solution because the divergence between species is not yet successful in delineating between all species (Rubinoff, 2006).

The use of new approach DNA barcodes to highlight areas of traditional taxonomy that should be reevaluated has been gaining popularity in recent years (Kerr *et al.,* 2009). Taxonomists, ecologists, conservationists, foresters, agriculturalists, forensic scientists, customs and quarantine officers have been using barcoding technique for plant identification. According to Hollingsworth *et al.,* (2011), it can be used in following situations: (a) geographically focused studies aiming to distinguishing among the diversity at a given site or region, where many of the samples are not necessarily closely related, and particularly where juvenile material and plant fragments require identifications; (b) species in trade, where the challenge is often to distinguish between a set of target species, and often distantly related potential substitutes or to identify members of higher taxonomic groups (e.g., family, genus) rather than particular species and (c) where the identification problem relates to unfamiliarity with a given species such that the user may have no idea even what family a given species belong to. Likewise, DNA barcoding can provide identifications where materials has been processed in one way or another, such as analyzing the diet of herbivores, food products or the components of herbal medicines (Valentini *et al.*, 2009; Jaakola *et al.*, 2010).

2.6.1.2 Biodiversity conservation

Another proposed use of DNA barcoding is to quickly catalogue all of the biodiversity on the planet before it disappears and to help determine where to focus conservation efforts (Rubinoff 2006a, 2006b; Langhoff *et al.,* 2009). There are about 29000 plant species protected by CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora)(http://www.cites.org/eng/disc/species.shtml, 2014), and developing effective methods to distinguish CITES listed from none CITES listed species is important for the conservation of endangered species. Primate Population is reduced in Africa by 90% because of bush meat hunting. DNA barcoding can be used law enforcement to bush meat traders in local markets which is obtained from bush meat (Kaur, 2015).(Ogden *et al.,* (2008) developed a Single Nucleotide Polymorphism (SNP) genotyping approach based on *matK* DNA barcodes to distinguish between Ramin (*Gonostylus*) species which are CITES

protected, and other confamilial species ornatomically similar but distantly related species. The level of biological diversity present in an environment can be quantified by either enumerating numbers of species (e.g., Simpson's diversity) or estimated evolutionary divergences among species in which genetic distances have been calculated. Although most measures of alpha and beta diversity across plant communities are based on numbers of species, DNA sequence data will provide an evolutionary dimension to diversity estimates that incorporate genetic distance among species (i.e., phylogenetic branch lengths). Evolutionary diversity, also called phylogenetic diversity, is correlated with, but not equivalent to, species richness. For example, geographic areas that harbor relatively few species may have high phylogenetic diversity if the species present are broadly dispersed across the Tree of Life. It has also been shown that a discontinuity between species richness and evolutionary diversity occurs when there are concentrations of closely related species in an environment. When compared with species richness in the same communities, these genetic measures can also be used to evaluate species boundaries, can serve as clues to assist in documenting new species, and can identify targeted habitats for conservation. . These applied uses of DNA barcodes for conservation and commercial purposes will undoubtedly increase in the future, especially as sequencing technology becomes simpler and less expensive (Kress *et al*., 2014).

2.6.1.3 Identifying pests and invasive species

It is often difficult to identify the larval stage of insects that are responsible for destroying crops. Once the type of pest is quickly identified, the farmer could proceed with treatment more rapidly and lose fewer crops (Mitchell, 2008). DNA barcoding can help in identifying pests in any stage of life making easier to control them saving farmers from cost of billion dollars from pest damage. The global *tephritid* barcoding initiative contributes to management of fruit flies by providing tools to identify and stop fruit flies at border (Kaur, 2015). Also, the rapid identifications provided by DNA barcoding could also be beneficial for managing invasive species. This technology would be especially useful at commercial ports and national borders, where a speedy identification of taxa could result in swift action that could prevent the spread of the invasive species (Mitchell, 2008).

2.6.1.4Angiosperm classification based on barcoding technology

DNA sequences generated from nuclear and chloroplast genomes have been widely used for phylogenetic analysis in plants (Hebert *et al.,* 2003). Many researchers have come to the

conclusion that phylogenies based solely on the barcoding region are inferior to phylogenetic signal and hence should include more sequences from various partial genomes. This is true of any study based on a small portion of sequence data from a single gene. Hence, phylogenetic analyses require sequence data from multiple unlinked genes (Mitchell, 2008). The Angiosperm Phylogeny Group, or APG, refers to an informal international group of systematic botanists who came together to try to establish a consensus view of the taxonomy of flowering plants (angiosperms) that would reflect new knowledge about their relationships based upon phylogenetic studies. The initial paper by the APG(1998) made angiosperms the first large group of organisms to be systematically re-classified primarily on the basis of genetic characteristics. Earlier classification been rejected because they were not phylogenetic, i.e. are not based on strictly monophyletic groups (i.e. groups which consist of all descendants of a common ancestor). It was superseded in 2003 by a revision, the APG II system, and then in 2009 by a further revision, the APG III system. These systems are molecular based on chloroplast and nuclear genes. The APG II system recognized 45 orders, five more than the APG I system and APG III recognized 415 families, 42 fewer than in the previous system on the basis of molecular phylogenetic classification (Angiosperm Phylogeny Group, 2009).

2.7Phytochemicals screening

Following descriptions of various phytochemicals are based on Khan, 2015.

2.7.1. Secondary metabolites

All those organic compounds present in plants and in animals that are not working in the normal growth, development or reproduction of organisms but produced in different metabolic processes are called secondary metabolites. Secondary metabolites are not essential for life as compared to primary metabolites, that the absence of secondary metabolites results not in failure of life, but in long-term impairment of the organism's survivability/fecundity or aesthetics, or perhaps in no significant change at all but it is useful for animal's ailments and normalizes the physiological abnormalities produced due to different diseases in animal bodies. Secondary metabolites are often very restricted to a particular set of species within a phylogenetic group. In broad sense, organic molecules may be classifed into; 1) Small molecules or micromolecules (alkaloids, terpenoids, glycosides, Phenols and Phenazene), 2) big small molecules (Polyketides, Non ribosomal peptides etc), 3) Non-small molecules or macromolecules (DNA, RNA, ribosome, polysacharides).

2.7.1.1 Small molecules

Alkaloids

Alkaloids are natural product that contains basic nitrogen atoms. The name of alkaloids derives its name from the "alkaline". Alkaloids are naturally synthesized by a large numbers of organisms, including animals, plants, bacteria and fungi. Alkaloids are a group of natural products (also called secondary metabolites). Alkaloids can be easily purified from various crude extracts by acid-base extraction. There are very many alkaloids which are toxic to other organisms. They often have some pharmacological effects and are used for the treatment of various diseases and recreational drugs. Some alkaloids are used as the local anesthetic and stimulant as cocaine. Some alkaloids have stimulant property as caffeine and nicotine compounds like morphine are used as the analgesic and quinine as the antimalarial drug. Almost all the alkaloids have a bitter taste.

Classification

Alkaloids may be classified in different groups on the bases of their structure formulas.

- **Pyridine group**: Nicotine alkaloid found in tobacco (*Nicotiana tabacum*) plantand Anabasine alkaloid found in the tree Tobacco (*Nicotiana glauca*) plant.
- **Pyrrolidine group:** Hygrine found in *Erythroxylum coca* leaves
- **Tropane group:** Atropine alkaloid found in *Atropa belladonna* and *Datura stramonium,* Cocaine alkaloid found in *Erythroxylum coca* leaves.
- **Indolizidine group:** one example is Swainsonine that was first obtained from a very small plants like pea (e.g. *Swainsona* sp. and *Astragalus* sp).
- **Quinoline group:** Quinine alkaloidsisolated originally from *Cinchona succirubra* and Strychnine alkaloids was obtained from the seeds of the *Strychnos nux vomica* tree.
- **Isoquinoline group:** The *Opium* alkaloids like narcotine, papaverine, narceine, morphine, codeine, heroine, sanguinarine, hydrastine, alkaloids like berberine, emetine, berbamine, oxyacanthine from *Berberis* species
- **Phenanthrene alkaloids:** *Opium* alkaloids like morphine, codeine, thebaine are included in this group.
- **Phenethylamine group:** Alkaloids found in many members of the Cactaceae like *Lophophora williamsii* and *Echinopsis pachanoi* i.e. Mescaline alkaloids etc, and some alkaloids found in *Ephedra vulgaris* i.e. ephedrine alkaloids etc are included in this group.
- **Indole group:** Serotonin is found in the enterochromaffin cells in the gut of animals, but also found in mushrooms and plants, including fruits and vegetables, Vinca alkaloids such as vinblastine, vincristine found in *Catharanthusroseus* etc.
- **Purine group**: Caffeine type of alkaloids are abundant in genus *Coffea Coffea canephora* (also known as *Coffea robusta*) and *Coffea arabica* are two speceis which have been grown for this purpose.

- **Terpenoid group:***Aconitum* alkaloids such as aconitine, Steroid alkaloids such as alkaloids found in *Solanum* i.e. solanine, solanidine and chaconine etc.

Terpenoids

The terpenoids sometimes called isoprenoids, are a class of natural products which are very similar to terpenes that have been derived from five-carbon isoprene units and can be interchanged in thousands of ways. Most of the terpenoids have multi cyclic structures that differ from one another by their functional groups and basic carbon skeletons. These types of natural lipids can be found in every class of living things, and therefore considered as the largest group of natural products

Classification

Terpenoids can be thought of as modified terpenes, where terpenes are hydrocarbons resulting from the combination of several isoprene units. The classification of terpenoids can be made according to the number of isoprene units used.

- **Hemiterpenoids:** Consist of a single isoprene unit. The only hemiterpene is the Isoprene itself, but oxygen-containing derivatives of isoprene such as isovaleric acid and prenol is classifed as hemiterpenoids.

- **Monoterpenoids**: Biochemical modifications of monoterpenes such as oxidation or rearrangement produce the related monoterpenoids. Monoterpenoids have two isoprene units. Monoterpenes may be of two types i.e linear (acyclic) or contain rings e.g. Geranyl pyrophosphate, Eucalyptol, Limonene and Pinene.

- **Sesquiterpenes:** Sesquiterpenes have *three isoprene* units e.g. Farnesyl pyrophosphate, Artemisinin, Bisabolol.

- **Diterpenes**: It is composed of four isoprene units and have the molecular formula $C_{20}H_{32}$. They are derived from geranylgeranyl pyrophosphate. There are some examples of diterpenes such as cembrene, kahweol, taxadiene and cafestol (precursor of taxol). Retinol, retinal, and phytol are the biologically important compounds while using diterpenes as the base. These three compounds are known to be antimicrobial and anti-inflammatory.

- **Sesterterpenoids:** Terpenoids having 25 carbons and *five isoprene* units are called sesterterpenoids.

- **Triterpenes:** It consist of *six* isoprene units e.g. squalene found in wheat germ and olives.

- **Tetraterpenoids:** It contain eight isoprene units which may be acyclic like lycopene, monocyclic like gamma-carotene, and bicyclic like alpha- and betacarotenes.

- **Polyterpenoids**: It consists of a larger number of isoprene units.

Glycosides

It is a group of natural product where a sugar group is directly bonded through its anomeric carbon to another group by an O-glycosidic bond or an S-glycosidic bond. The sugar group is then known as the glycone and the non-sugar group as the aglycone or genin part of the glycoside. The glycone can consist of a single sugar group (Monosaccharide) or several sugar groups (oligosaccharide).

Classification

Glycosides may be classified in three ways *Viz.*

i) **Type of glycone**: If the glycone group of a glycoside is glucose, then the molecule is a glucoside, if it is fructose, then the molecule is a fructoside; if it is glucuronic acid, then the molecule is a glucuronide etc. In the body, toxic substances are often bonded to glucuronic acid to increase their water solubility,the resulting glucuronides are then excreted.

ii) **Type of glycosidic bond**: It is classified as α-glycosides or β-glycosides depending on bond geometry that whether the glycosidic bond lies "below" or "above" the plane of the cyclic sugar molecule. On the bases of this particular geometry some enzymes like α-amylase can only hydrolyze α-linkages while others like emulsin, can only affect β-linkages

iii) **Type of aglycone**. Glycosides are also classified according to the chemical nature of the aglycone e.g.

- **Alcoholic glycoside**: Salicin is an example of an alcoholic glycoside which has been isolated from the genus *Salix*. Salicin is converted to salicylic acid in the body, which is closely related to aspirin and has analgesic, antipyretic and antiinflammatory effects.

- **Anthraquinone glycosides**: They are present in senna, rhubarb and aloes, they have a laxative effect.These glycosides contain an aglycone group that is a derivative of anthraquinone.

- **Coumarine glycosides**: Psoralin and corylifolin are obtained from dried leaves of *Psoralea corylifolia* and the aglycone is coumarin. Apterin is a coumarine glycosides which is reported to dilate the coronary arteries as well as block calcium channels.

- **Cyanogenic glycoside:** The aglycone contains a cyanide group, and the glycoside can release the poisonous hydrogen cyanide if acted upon by some enzyme. They are stored in the vacuole but if the plant is attacked they are released and become activated by enzymes in the cytoplasm. These remove the sugar part of the molecule and release toxic hydrogen cyanide. Storing them in inactive forms in the cytoplasm prevents them from damaging the plant under normal conditions. An example of these is amygdalin from almonds. They can also be found in the fruits (and wilting leaves) of the rose family (including cherries, apples, plums, almonds, peaches, apricots, raspberries and crabapples).

- **Flavonoid glycosides:** In this type of glycosides the aglycone units are flavonoids e.g. Hesperidin (aglycone: Hesperetin, glycone: Rutinose), Rutin (aglycone: Quercetin, glycone: Rutinose), Querctrin (aglycone: Quercetin, glycone: Rhamnose).

- **Phenolic glycosides:** The aglycone is a simple phenolic structure e.g. Arbutin found in *Arctostaphylos uva-ursi.*

- **Saponin glycosides:** The characteristic of saponin glycoside is that they normally produce soap-like foaming when shaken in aqueous medium, and structurally saponin gycosides are composed of one or more

hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. Saponin glycosides are found in liquorice (*Glycyrrhiza glabra).*

- **Steroidal glycosides:** The aglycone part is a steroidal nucleus. The glycosides of *Digitalis, Scilla* and *Strophanthus*. These glycosides are more effective in heart diseases.

- **Steviol glycosides:** The glycosides found in *Stevia rebaudiana bertoni* are about 300 times sweeter than sucrose. Stevioside and rebaudioside A, are used as natural sweeteners in many countries.

- **Thioglycosides:** These glycosides contain sulfur e.g. sinigrin and sinalbin found in black and white mustard respectively.

Phenols

Phenols or Phenolic are hydroxyl group (-OH) containing class of chemical compounds where the (-OH) bonded directly to an aromatic hydrocarbon group. Phenol (C_6H_5OH) is considered the simplest class of this group of natural compounds. Other examples are Resveratrol, Polyphenols (flavonoids and tannins), Gallic acid, Eugenols etc.

Phenazines

It is also called azophenylene, dibenzo-p-diazine, dibenzopyrazine, and acridizine, is a dibenzo annulated pyrazine and the parent substance of many dyestuffs, such as the eurhodines, toluylene red, indulines and safranines. Pyocyanin is a toxic blue crystalline Pigment($C_{13}H_{10}N_2O$) that is formed in the metabolism of a bacterium of the genus Pseudomonas (*P. aeruginosa*), gives a bluish tint to pus infected with this organism, is a quinone imine related to phenazine, and has antibiotic activity especially toward gram positive bacteria.

2.7.1.2 Big “small molecules”

Polyketides

Polyketides are synthesizedlike a process of fatty acid synthesis that are synthesized from fatty acid. The polyketides are also biosynthesized by the polymerization of propionyl and acetyl subunits. They are also the building blocks for variety of natural products or are further derivatized. Examples are

- **Macrolides:** It includes Picromycin, the antibiotics of erthromycin A, Clarithromycin and azithromycin, the immunosuppresent tacrolimus (FK506).
- **Polyene antibiotics**: It include Amphotercin which was isolated from *Streptomyces nodosus*, a filamentous type bacterium and use as antifungal drug.
- **Tetracyclines**: The tetracycline family is broad-spectrum polyketide antibiotic produced by the *Streptomyces* genus of Actinobacteria, indicated for use against many bacterial infections.
- **Acetogenins:** It includeAnnonacin found in fruits such as the guanabana and Uvaricin is a bis(tetrahydrofuranoid) fatty acid lactone present in the roots of *Uvaria accuminata*.

Nonribosomal peptides

It usually produced by microorganisms like bacteria and fungi. Nonribosomal peptides are also found in higher organisms, such as nudibranchs. Nonribosomal peptides are synthesized by nonribosomal peptide synthetases, which, unlike the ribosomes, are independent of messenger RNA. Example are

- **Vancomycin:** It is produced fromthe organism *Amycolatopsis orientalis*. It is a glycopeptide type antibiotic and used for Gram-positive bacteria produced prophylaxis and treatment of infections. It is very important antibiotic and not always used but only in cases where the other antibiotics had failed. It is therefore named as a drug of "last resort".

- **Thiostrepton:** Cyclic oligopeptide antibiotic, derived from several strains of Strepromyces, such as *Streptomyces azureus* and *Streptomyces laurentii*.

2.8 Biological assays

Bioassay is defined as the estimation of the potency of an active principle in a unit quantity of preparation or detection and measurement of the concentration of the substance in a preparation using biological methods (i.e. observation of pharmacological effects on living tissues, microorganisms or immune cells or animal) (Goyal, 2008).Plant originated natural products have played and will continue to play important role in pharmaceutical industry to discover and deliver chemicals and biological entities for the treatment of various diseases (Gupta, 1994).

Documentation of antimicrobial properties and toxicity of medicinal plants is essential to build a comprehensive database from which it may be potential to search new leads in development of drugs (Nguta *et al*., 2013).

2.8.1Antibacterial Susceptibility Assay

Plants, as the source of medicine, have been playing an important role in the health services around the globe. About three quarters of the world's population relies on plant and their extracts for health care. A good number of our population particularly those living in rural areas depend largely on herbal remedies for the treatment of different types of diseases. It indicates the importance of the individual plants in the health care system (Subedi *et al.,* 2012).

Plant-derived materials or products with therapeutic properties are known as herbal medicines, they may contain processed or raw ingredients from one or more plants that are beneficial for human health. Medicinal plants are important with respect to new drug and pharmacological research and development. They are widely used and accepted as home remedies and raw materials for the pharmaceutical industries (Walter *et al*., 2011).

2.8.2 Brine –shrimp cytotoxicity Assay

The brine shrimp lethality bioassay is rapid (24 h), simple (e.g., no aseptic techniques are required), easily mastered, inexpensive, and requires small amounts of test material (2-20 mg or less) (Ghisalberti, 1993). The bioassay has a good correlation with cytotoxic activity in some human solid tumors and with pesticidal activity (McLaughlin *et al.,* 1998). This test was proposed by Michael *etal.*(1956) and modified by Mayer *et al*., (1982). Since its introduction, this *in vivo* lethality test has been successively employed for providing a frontline screen that can be backed up by more specific and more sophisticated bioassays once the active compounds have been isolated. This test is based on the potential of *Ephedra gerardiana* methanol and hexane extract to become lethal to *Artemia Salina* nauplii due to its toxic expression. According to Meyer *et al*., 1982, extracts derived from natural products which have LC50 ≤ 1.0 mg/mL are known to possess toxic effects.

Cancer is the leading cause of death in the United States, where one in four deaths is due to cancer as well as it is a serious health problem and is the major cause of human mortality all over the world. Recently, there has been difficulty in the treatment of certain forms of cancer due to their resistance to some of the drugs in the market (Mohammed, 2009). Therefore, it has become imperative for natural product chemists to intensify the search for new anticancer and antitumor drugs, which are not only very potent but affordable (Musa, 2012). One mode of cancer treatment is through the use of cytotoxic drugs. Cytotoxic drugs have the potential to kill the cancerous cells and are usually developed after initial screening of thousands of lead compounds from various sources.

Among the many recent advances in cancer chemotherapy, it has been noticed that plant derived compounds play an important role in development of chemotherapeutic drugs. The earth is a vast repository of medicinally important plants. It is imperative that such plants must be subjected to exhaustive studies for screening the bioactive compounds (Shreeshma & Nair, 2014).

Assay systems are available which give a preliminary idea on the cytotoxicity and therefore the anticancer potential of plant extracts. Among the available cytotoxicity screening assays, Brine Shrimp Lethality Assay (BSLA) appears to be the most rapid (24 hours), simple (no aseptic techniques are required), easily mastered, and inexpensive method. Moreover, it requires only small amount of test material (2 or 20 mg or less). BSLA has been routinely used in the primary screening of the extracts as well as isolated compounds to provide an

indication of possible cytotoxic properties of the test materials (Shreeshma & Nair, 2014).This method involves the exposure of newly hatched brine- shrimp nauplii to the palnt extract or the isolated compounds of known dose levels.After 24 hours, the survivors are counted and the percentage death is calculated. The biological activity is evaluated on the basis of toxicity towards nauplii. The LC_{50}(lethal concentration 50) value and 95 %CI (Confidence Interval) are computed using the probit analysis. The LC_{50} value is the log concentration for 50% survival and is calculated by following formula:

$$Y = \alpha + \beta X \quad (1)$$

$$X = (Y - \alpha)/\beta \quad (2)$$

$$\alpha = \frac{1}{n}(\sum y - \beta \sum x) \quad (3)$$

$$\beta = \frac{\sum xy - \left[\frac{\sum x . \sum y}{n}\right]}{\sum X^2 - \frac{[\sum (x)^2]}{n}} (\sum y - \beta \sum x) \quad (4)$$

Where,

X=$\log_{10}$ of constituents in ($\mu g/mL$)i.e. $\log_{10}10$, $\log_{10}100$ and $\log_{10}1{,}000$

Y= probit for the average survivor of all the replicates

n=number of dose levels

$\sum x$= sum of the log of doses$\mu g/mL$

$\sum y$= sum of the responses

$\sum xy$= sum of the value of xy.

$\sum x^2$ = sum of the values of x^2.

LC_{50} = Antilog (X) (5)

95% CI - $LC_{50} \pm 1.96\left(\frac{\sigma}{n}\right)$ (6)

Where

Standard deviation $(\sigma) = \frac{1}{n-1}\left[\sum X^2 - \frac{(\sum X)^2}{n}\right]$ (7)

The extracts/isolated compounds having LC_{50} value (µg/mL) less than 1,000 µg/ml are considered to be toxic or pharmacologically active and those greater than value 1,000 µg/mL are non- toxic or pharmacologically inactive.

CHAPTER 3 – MATERIALS AND METHODS

Materials

3.1 Plant materials

A total of nine samples of different species of Rhododendrons and whole plants of *Ephedra gerardiana*was collected from high altitude of Sagarmatha National Park, Nepal during December, 2013. The collected samples were photographed and altitude, latitude & longitude of the collection sites were measured using GPS and plants were designated generic accession code according to the criteria of Molecular biotechnology unit, Nepal Academy of Science and Technology (NAST). The leaf samples were placed in lens paper pouches which were stored in dried silica gel beads in air tight plastic container. All these samples were brought to Molecular Biotechnology Unit Laboratory of NAST for subsequent molecular analysis. The collected samples were Identified by taxonomist from KATH, National Herbarium and Plant Research Laboratory, Godawari.

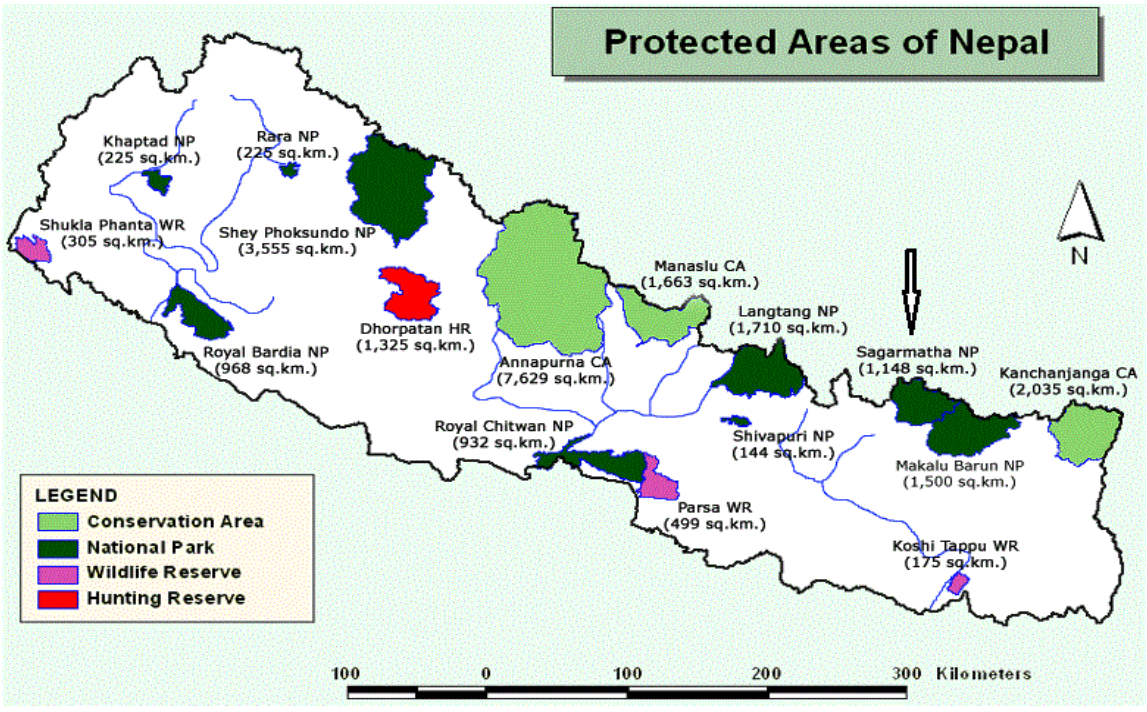


Figure 3.1 *Rhododendron species* and *Ephedra gerardiana* sampling sites of present investigation (highlighted & arrowed).

Table 3.1 Details of samples collected from different altitudes of Sagarmatha National Park, Nepal.

S.N	Call No.	Name	W.P.	Latitude	Longitude	Altitude(m)
1	SNP 137	*R.barbatum*		27°83′74.8″N	0867°6′72.2″E	3810
2	SNP 240	*R.arboreum*	217	274°8′40.0″N	086°41′49.1″E	3524
3	SNP 269	*R.setosum*	18	27°49′19.6″N	086°43′22.5″E	3780
4	SNP271	*R.anthopogon*	20	27°49′13.1″N	086°43′22.3″E	3780
5	SNP 273	*R.campylocarpum*	21	27°49′09.7″N	08°43′24.4″E	3852
6	SNP 283	*R.campanulatum*		27°83′74.8″N	0867°6′72.2″E	3802
7	SNP 318	*R.nivale*	37	27°57′32.0″N	086°48′52.5″E	5042
8	SNP 319	*R.lepidotum*	37	27°57′32.0″N	086°48′52.5″E	5042
9	SNP 323	*R.hodgsonii*		2783781°N	086.76675°E	3802

Table 3.2 Information of Sample *E. gerardiana* collected:

Call No.	Name	Family	Lattitude	Longitude	Altitude(m)	Parts of the plant collected
SNP299	*E.gerardiana*	Ephedraceae	27°54′40.7″N	086°48′32.8″E	4402	Whole plant

Methodology

3.2 DNA extraction by modified Doyle and Doyle Extraction Method

For DNA extraction from various Rhododendron spp. modified Doyle & Doyle, 1990 technique adapred by Kunming Insitute of Botany (KIB) Kunming, China was used.

The leaf samples (approximately 100mg) were sterilized using 0.2% sodium hypochlorite (NaOCl) and were left to dry to sometime. The samples were ground in sterilized mortar and pestle in presence of liquid nitrogen. The ground samples were treated with extraction buffer (1000µl) and transferred to sterile Eppendorf tubes (1.5ml). The mixtures were then incubated at 65°C for 15 minutes. Thereafter, the tubes were centrifuged at 11000 rpm (~13000g) for 5 minutes and the supernatants were transferred to a clean sterile microfuge tubes and equal volumes (~500 µl) of chloroform: isoamyl alcohol (24:1) were added to each tube The solutions were mixed gently by inversions several times. Tubes were then centrifuged at 11000 rpm for 1 minute at 25 ^{o}C and the upper aqueous phase were transferred to clean eppendorf tubes and re-extracted with equal volumes of chloroform: isoamyl alcohol (24:1). Tubes were centrifuged for 1 minute at 11000 rpm and the upper aqueous layers were transferred to new tubes. Each sample was then treated with 1/10th volume (approximately 50 µl) of ammonium acetate (7.5M) followed by addition of equal volume (~500 µl) of ice-cold (-20°C stored) absolute ethanol. Then the tubes were placed overnight at -20°C to allow precipitation of DNA. Next day tubes were centrifuged at 11000 rpm for 10-15 minutes at 4°C. The supernatants were discarded and the DNA pellets were quickly washed twice with ice cold 70% ethanol (-20°C stored). In order to get rid of unwanted salts, samples were briefly spun at 11000 rpm for 1 minute and excess alcohol was pipetted off. Then pellets were dried in air for 5 minutes in laminar air flow and were re-suspended in TE buffer (with RNase) and stored at -20°C.

3.3 DNA Quantification

DNA quantification and purity assessment was carried out using spectrophotometric method (Biophotometer, Eppendrof-AG223331, Germany).

3.4 Gel Electrophoresis

The PCR amplified products of DNA Barcode-PCR (PCR amplified products using ITS, *matK, rbcL and trnH-psbA* markers) and extracted DNA were analyzed on a 1.5% Agarose gel in TAE buffer (1X) at 50V for two hour using EMBI TEC (Sandiago, CA) gel tank. Total volume loaded in well was 4µl DNA and 1µl GLB (6X) for DNA analysis and 10µl PCR product and 2µl GLB (6X) for PCR product analysis. The Ethidium Bromide (EtBr) (10mg/mL; Promega) was added during gel preparation at a concentration of 5µl per 100mL

agarose gel (Sambrook and Russell, 2001). After gel run, the gels were visualized on a gel documentation system (IN GENIUS, Syngene Bioimaging, UK).

3.5 Amplification of nrITS, *matK, rbcL* and *trnH-psbA* regions

All the ITS, matk, rbcl and trnH-psbA sequences were amplified using 2X Master Mix (Promega). The components of Master Mix (Promega) were as tabulated in Table 3.3.

Table 3.3 Components of 2X Master Mix obtained from Promega

S.N.	**Components**	**Concentration**
1.	*Taq* DNA Polymerase (pH 8.5)	50 U/mL
2.	dNTPs (dATP, dGTP, dCTP, dTTP)	400 μM (each)
3.	$MgCl_2$	1.5 mM

Four standard DNA barcode regions were chosen for the study. Two of the regions were coding sequences (*matK* and *rbcL*) and are considered core barcodes for flowering plants as suggested by CBOL Plant working group (2009) while two were non-coding intergenic spacers (ITS and *trnH-psbA)*. The primers used for amplification of these loci are summarized in the Table 3.5.

Table 3.4 Primers detailed used in the amplification of ITS, *matK, rbcL* and *trnH-psbA*regions**.**

Locus	**Primers**	**Sequence (5'-3')**	**Reference**
nrITS	ITS 5 (F) ITS 4 (R)	GGAAGGAGAAGTCGTAACAAGG TCCTCCGCTTATTGATATGC	(White *et al.*, 1990)
matK	3F_KIM (F) 1R_KIM (R)	CGTACAGTACTTTTGTGTTTACGAG ACCCAGTCCATCTGGAAATCTTGGTT C	Cuenoud *et al.,* 2002
rbcL	1 (F) 724 (R)	ATGTCACCACAAACAGAAAC TCGCATGTACCTGCAGTAGC	Kress and Erickson, 2007)
trnH-psbA	psbA3_f (F) trnHf_05 (R)	GTTATGCATGAACGTAATGCTC CGCGCATGGTGGATTCACAATCC	(Sang *et al.*, 1997) (Tate and Simpson, 2003)

Amplification was done using 2X PCR Master Mix (Promega, USA). PCR program and the PCR components used were similar for *matK, rbcL* and *trnH-psbA* while the program for nrITS was different. The PCR amplification was carried out in 25 µL reaction mixture. In case of $MgCl_2$ concentration for *matK, rbcL* and *trnH-psbA* amplification, a further 1 mM was added to original 1.5mM from master mix making the final concentration 2.5 mM, template DNA 25 ng and primer 0.4 pm. Similarly, for ITS region $MgCl_2$ concentration 2 mM was added to original volume 1.5 mM from master mix making 3.5, template DNA 25 ng and primer 0.8 pm. The amplification of DNA templates were done in BioER thermal cycler (BioER, China). The different components used for different barcode loci are tabulated in Table 3.6.

Table 3.5 Different reaction conditions used for amplification of different barcode loci

S.N	**Barcode loci**	**Components**	**Concentration used**
1.	*matK, rbcL, trnH-psbA*	2X Master Mix	1X (12.5 µL)
		Primers (Table 3.7)	0.4 pm
		tDNA	25 ng
		$MgCl_2$	2.5 mM
2.	nrITS	2X Master Mix	1X (12.5 µL)
		Primers (Table 3.7)	0.8 pm
		tDNA	25 ng
		$MgCl_2$	3.5

The cycling conditions used for amplification of different barcode loci are presented in Table 3.6.

Table 3.6 Different cycling conditions used for amplification of different barcode loci

S.N.	Barcode loci	Cycling conditions	No. of cycles
1.	*Matk* *rbcL* *trnH-psbA*	94°C – 2 mins	35 Cycles
		94 °C – 35 secs	
		52 °C – 40 secs	
		72 °C – 55 secs	
		72 °C – 7 mins	
		4 °C – hold	
2.	ITS	94°C – 3 mins	Step 1
		50 °C – 1 min	
		72 °C – 2 mins	
		94 °C – 30 secs	39 cycles
		50 °C – 30 secs	
		72 °C – 75 secs	
		72 °C – 5 mins	
		25 °C – 2 mins	
		4 °C – 20 mins	

3.6DNA barcoding and phylogenetic analysis of *Rhododendron spp.*

3.6.1 DNA Sequencing

All the total of nine species were selected for DNA sequencing of four different loci ITS, *matK, rbcL and trnH-psbA.*

A total of 20 µL each of PCR amplified DNA samples were sent for bidirectional sequencing to Macrogen (Macrogen Inc.), South Korea.

3.6.2 Sequence Analysis

The sequence data generated by Macrogen, Korea after sequencing of all the samples were assembled using CodonCode Aligner version 4.0.4 software (CodonCode Corporation

Centerville, MA 02632, USA). Consensus sequences were generated for each locus of individual sample.

Using the consensus sequences, the most similar sequences were searched in the National Center for Biotechnology Information (NCBI) nucleotide database using Basic Local Alignment Search Tool (BLAST)(Altschul *et al.*, 1990). The most similar sequences were aligned with the assembled sequences using CodonCode Aligner version 4.0.4 and adjusted manually. Multiple sequence alignment was performed using ClustalW (Thompson *et al.*, 1994)option of Molecular Evolution Genetics Analysis (MEGA) version 6.0(Tamura *et al.*, 2011). The intraspecific and interspecific variation of each barcoding region was characterized by calculating Kimura 2-parameter (K2P) distances in MEGA v.6.0.

3.6.3 Phylogenetic Analysis

Maximum Parsimony (MP) trees based on *matK, rbcL, trnH-psbA* and ITS sequences were constructed using MEGA v. 6.0 with 1000 bootstrap replications to determine clade support. Branch swapping algorithm used was Subtree-Pruning and Regrafting (SPR) algorithm. Condensed tree with 50% cutoff value was used to depict the phylogenetic relationship.

3.6.4 Model based Phylogeny

For model based phylogeny, the selection of best-fit model of nucleotide substitution for each of four loci ITS, *matK, rbcL* and *trnH-psbA*was done using jModelTest v. 2.1.4 (Darriba *et al.*, 2012) using Bayesian Information Criterion(BIC). Maximum likelihood scores were calculated using 88 nucleotide substitution models in jModelTest. On the basis of selected nucleotide substitution models, the jModelTest v. 2.1.4 strict consensus tree were constructed under 95% confidence interval.

3.7Phytochemical analysis of *Ephedra gerardiana*

3.7.1Preparation of the plant material

The collected plant material were cleaned off mud, fungi and any unwanted materials, then air or shade dried at 32-35^{o}C for six days to remove all their moisture. The dried plant material was powdered with the help of grinder and the fine powder was collected on sterile and dry polyethylene bag for extraction**.**

3.7.2Preparation of plant extracts and extract dilution

Extraction of compounds from *E.gerardiana* was carried out using hexane and methanol solvent. About 100 grams of fine powder of plant sample was taken and dissolved in 500 ml of 100 % hexane and left to percolate for 48 hours. The solvent was filtered and hexane was evaporated on the Rotatory Evaporator under the vacuum at the room temperature till the solid mass was obtained. Obtained solid mass was weighed carefully to express the gram of extract extracted per 100 grams of the plant powder. The same process was repeated for five times of the same extracted samples for both methanol and hexane. The extracts were kept at 4^{o}C for further analysis.

3.8Phytochemical Screening

The different types of phytochemical constituents present in methanol and hexane extracts were identified by the colour reactions with different reagents following the phytochemical screening method developed by (Harborne, 1973; Trease and Evans, 1989; Sofowora 1993).

3.8.1Tests Carried Out in the Methanol Extract

Test for Basic alkaloids

A pinch of plant extract was dissolved in 4 mL of 2 %(V/V) hydrochloric acid and filtered. The filtrate was used for following tests to assess the presence of alkaloids. The filtrate was equally divided into 4 different test tubes.

(A)Mayer Test: The first test tube solution was treated with 3 drops of Mayer's reagent. A colorless solution obtained indicated absence of basic alkaloids.

(B)Wagner test: The second test tube solution was treated with Wagner's reagent. A reddish colour obtained indicated absence of basic alkaloids.

(C)Dragendroff test: The third test tube solution was treated with Dragendroff's reagent. A colourless solution obtained indicated absence of basic alkaloids.

(D)Hager test: The fourth test tube solution was treated with Hager's reagent. A colouless solution obtained indicated absence of basic alkaloids.

Test for Carbohydrate

A pinch of plant extract was dissolved in 2 mL distilled water and filtered. The filtrate was used for the presence of Carbohydrate. The filtrate was equally divided into 2 test tubes,

(A)Molisch test: The first test tube solution was treated with few drops of molisch's reagent. A violet ring at the junction between two layers formed indicated the presence of Carbohydrate. This was followed by addition of 1 mL of conc.H_2SO_4 to the side of the test tube. The mixture was then allowed to stand for five minutes. A reddish violet colour at the interphase of the two layers formed indicated presence of carbohydrate.

(B)Test for reducing compounds (Fehling's test): A pinch of plant extract dissolved to about 2 mL of the methanol, concentrated and mixed well in distilled water. To this 1 mL of Fehling's reagent (equal volume of Fehling's solution A & B) was added. The content was warmed over a boiling water bath for about 10 minutes. Brick red precipitate was deposited at the bottom of the test tube indicated the presence of reducing compounds.

Test for flavonoids

(A)Lead acetate test: A pinch of extract was treated with methanol and mixed with few drops of basic lead acetate solution. A yellow precipitate formed indicated the presence of flavonoids.

(B)Shinoda test: A pinch of extract was dissolved in 1 mL methanol and treated with a piece of magnesium and few drops of conc.HCl (5 drops).A Pinkish red colour obtained indicated the presence of flavone aglycones.

(C) Shibata test: A pinch of extract was dissolved in 1 mL methanol (MeOH) and treated with one small spatula of zinc and few drops (5 drops) of conc.HCl. A pinkish red colour obtained indicated the presence of flavone aglycones.

Test for Lactones

Baljel test: A pinch of extract mixed with 1 mL solution of sodium picrate. A yellow orange colour obtained indicated presence of lactones.

Test for Glycosides

Alkaline reagent test: A pinch of extract is dissolved in 1 mL methanol and to this solution 1 mL of 25% NH_4OH was added. A cherry red colour obtained indicated the presence of glycosides.

Test for Oxalates: A pinch of extract was mixed with 1 mL of ethanol and few drops of glacial acetic acid. A colouless solution obtained indicated absence of oxalates.

Test for Quinones: A pinch of extract was treated with 1 mL conc HCl. A yellow colour obtained indicated presence of quinones.

Test for steroids

Salkowski test: A pinch of extract was dissolved in 1 mL of $CHCl_3$ and shaken with equal volume of conc.H_2SO_4. A red colour obtained on standing indicated presence of steroids.

Test for terpenoids

Tschugajen test: A pinch of extract was treated with 1 mL of $CHCl_3$ and excess of acetyl chloride and pinch of Zinc chloride was added. Upon warming eosin red colour obtained indicated presence of terpenoids.

Test for Tannins and polyphenols

Braymer test: A pinch of extract was mixed with about 2 mL of water and heated on water bath. The mixture was then filtered and few drops (5 drops) of 1 % $FeCl_3$ was added. A dark green colour with bubbles obtained indicated the presence of tannins and polyphenols.

Test for Proteins and amino acids

Xanthoproteic test: A pinch of extract was heated with 1 mL of Conc.HNO3. A yellow colour obtained indicated presence of proteins.

Test for Saponins

Froth test: A pinch of extract was shaken with 1 mL of distilled water and then heated to boil and was vigorously shaken for a stable persistent froth. Frothing (creamy mass of small bubbles) of 1 cm layer obtained indicated presence of saponins.

Test for Fatty acids

Spot test: A pinch of extract was mixed with hexane. The supernatant solution was spotted on a filter paper and the solvent was allowed for evaporation. No transparent spot obtained indicated absence of fatty acids.

Test for Anthraquinones

Borntiager test: A pinch of extract was boiled with 1 mL of 10 % HCl for few minutes, filtered and cooled. Equal volume (1 mL) of $CHCl_3$ was added to the filtrate. Few drops of 10 % $NH_3$was added and mixture was heated on water bath for five minutes. A green colour with bubbles like structure obtained indicated absence of anthraquinones.

Test for Leuconanthocyanins

A pinch of extract was boiled with 1 mL distilled water and cooled then 1 ml of isoamyl alcohol was added. A colourless upper layer obtained indicated absence of leucoanthocyanins.

3.8.2Tests carried out in the hexane extract

Test for Basic alkaloids

A pinch of hexane extract was dissolved in 4 ml of 2 %(V/V) hydrochloric acid and filtered. The filtrate was used to following tests for the presence of alkaloids. The filtrate was equally divided into 4 different test tubes.

(A)Mayer Test: The first test tube solution was treated with 3 drops of Mayer's reagent. A colorless solution was obtained indicated absence of basic alkaloids.

(B)Wagner test: The second test tube solution was treated with Wagner's reagent. A reddish colour obtained indicated absence of basic alkaloids.

(C)Dragendroff test: The third test tube solution was treated with Dragendroff's reagent. A colourless solution obtained indicated absence of basic alkaloids.

(D)Hager test: The fourth test tube solution was treated with Hager's reagent. A colouless solution obtained indicated absence of basic alkaloids.

Test for Carbohydrate

Plant extract was dissolved in 2 mL distilled water and filtered. The filtrate was used for the presence of Carbohydrate. The filtrate was equally divided into 2 different test tubes,

(A)Molisch test: The first test tube solution was treated with few drops of molisch's reagent. A violet ring at the interphase of the two layers formed indicated the presence of Carbohydrate. This was followed by addition of 1 mL of conc.H_2SO_4 to the side of the test tube. The mixture then allowed to stand for five minutes. A reddish violet colour at the interphase of the two layers formed indicated presence of carbohydrate.

(B)Test for reducing compounds (Fehling's test): About 2 mL of the hexane extract was concentrated and mixed well in distilled water. To this 1 mL of Fehling's reagent (equal volume of Fehling's solution A & B) was added. The content was warmed over a boiling water bath for about 10 minutes. Brick red precipitate was deposited at the bottom of the test tube indicated the presence of reducing compounds.

Test of flavonoids

(A)Lead acetate test: A pinch of extract was treated with methanol and mixed with few drops of basic lead acetate solution. A yellow precipitate formed indicated the presence of flavonoids.

(B)Shinoda test: A pinch of extract was dissolved in 1 mL methanol and treated with a piece of magnesium and few drops of conc.HCl (5 drops).A colourless solution obtained indicated the absence of flavone aglycones.

(C)Shibata test: A pinch of extract was dissolved in 1 mL methanol and treated with one small spatula of zinc dust and few drops (5 drops) of conc.HCl. A colourless solution obtained indicated the absence of flavone aglycones.

Test for Lactones

Baljel test: A pinch of extract was mixed with solution of sodium picrate. A yellow orange colour was obtained indicated presence of lactones.

Test for Glycosides

(A)Alkaline reagent test: A pinch of extract is dissolved in 1 mL methanol and to this 1 mL of 25% NH_4OH was added. A cherry red colour obtained indicated the presence of glycosides.

Test for Oxalates:A pinch of extract was mixed with 1 mL of ethanol and to this few drops of glacial acetic acid was added. A colouless solution was obtained indicated absence of oxalates.

Test for Quinones: A pinch of extract was treated with conc HCl. A colourless solution was obtained indicated absence of quinones.

Test for steroids

Salkowski test:A pinch of extract was dissolved in 1 mL of $CHCl_3$ and shaken with equal volume (1 mL) of conc.H_2SO_4. A red colour was obtained on standing indicated presence of steroids.

Test for terpenoids

Tschugajen test:A pinch of extract was treated with 1 mL of $CHCl_3$ and excess of acetyl chloride and pinch of Zinc chloride was added. Upon warming about five minutes eosin red colour obtained indicated presence of terpenoids.

Test for Tannins and polyphenols

Braymer test: A pinch of extract was mixed with water and heated on water bath for five minutes. The mixture was then filtered and few drops (5 drops) of 1 % $FeCl_3$ was added. A golden yellow colour obtained indicated absence of tannins and polyphenols.

Test for Proteins and amino acids

Xanthoproteic test: A pinch of extract was heated with 1 mL of Conc.HNO_3. A yellow colour obtained indicated presence of proteins.

Test for Saponins

Froth test: A pinch of extract was shaken with 1 mL of distilled water and then heated to boil and was vigorously shaken for a stable persistent froth. Frothing (creamy mass of small bubbles) of 1 cm layer obtained indicated presence of saponins.

Test for Fatty acids

Spot test: A pinch of extract was mixed with hexane. The supernatant solution was spotted on a filter paper and the solvent was allowed for evaporation. No transparent spot obtained indicated absence of fatty acids.

Test for Anthraquinones

Borntiager test: A pinch of extract was boiled with 1 mL of 10 % HCl for five minutes, filtered and cooled. Equal volume (1 mL) of $CHCl_3$ was added to the filtrate. Few drops of 10 % $NH_3$was added and mixture was heated on water bath for five minutes. A green colour with bubbles like structure obtained indicated absence of anthraquinones.

Test for Leuconanthocyanins

A pinch of extract was boiled with distilled water and cooled.1 mL of isoamyl alcohol was added. A red colour of upper layer obtained indicated presence of leucoanthocyanins.

Test for anthracenosides:A pinch of extract was mixed with ether (1 mL), Conc. H_2SO_4 (1 mL) and 1 mL of 25 % ammonia solution.A cherished-red colour on the top layer obtained indicated presence of anthracenosides.

3.7.4Antibacterial Susceptibility Assay

Antibacterial susceptibility assay of the plant extracts to evaluate the ability of killing or inhibiting the growth of the pathogenic organisms was carried out following agar well

diffusion method (Dingle *et al.*, 1953; Perez *et al*., 1990).It provides the rationale for the selection of the plants extracts containing potential antimicrobial compounds for further processing. In this study we have used the residue obtained after complete evaporation of the solvent from the methanol and hexane extracts of the plants materials for evaluation of their antibacterial property through determination of Zone of Inhibition (ZOI) against the tested organisms.

3.7.4.1 Sterilization of the apparatus

All the required glasswares and micropipette tips were autoclaved at 15 psi pressure for 15 minutes prior to use.

3.7.4.2 Bacterial strain and standard cultures

Seven different pathogenic bacterial strains, *Viz.*Gram – negative *Escherichia coli* (ATCC 25922), *Klebsiella Pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14038), and Gram- positive *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* and *Micrococccus luteus*were considered for the antimicrobial assay. The pure cultures were streaked in the nutrient agar plate for sub culture and incubated at 37 ^{o}C for 24 hours. Thus developed each pathogenic bacterial colony were transferred into a separate nutrient broth by using a sterile loop and incubated for 24 hours at 37 ^{o}C.

3.7.4.3Preparation of inocula

Selected bacterial strains from 24-hour oldcultures were mixed with nutrient broth (SIGMA) until a McFarland turbidity standard [10^{6} colony forming unit (CFU) ml^{-1}] was obtained. Then this inoculum was used to seed nutrient agar.

3.7.4.4Preparation of sample solution

0.05 g each of both samples (namely hexane extract, methanol extract) were separately transferred into clean, sterile vials aseptically and DMSO (1 mL) was added to make 50 mg/mL sample solutions.

3.7.4.5Screening and determination of antibacterial activities

Standard agar well diffusion assay was used for screening of antibacterial activities of the plant extract. Sterile cotton swab was dipped in each standard pure culture of seven tested organism and streaked all over the surface of the solidified agar medium by rotating the plates through angle of 60 º.The plates were left to dry for few minutes at room temperature inside laminar air flow hood. With the help of sterile cork borer having 6 mm diameter, the required number of wells were prepared in each nutrient agar plate and labelled properly. Now, the samples solutions (50 µl of each) were charged into the wells and solvent DMSO was used as negative control and Gentamycin antibiotic disc as positive control in one well of the plate separately. The plates were left for sometimes to diffuse solution in the media and then incubated at 37 ºC for 24 hours. After incubation, the plates were observed for Zone of inhibition (ZOI) produced due to antibacterial activity of the samples.

3.7.5 Brine – shrimp bioassay

3.7.5.1Preparation of the artificial sea water

The artificial sea water was used for the hatching of brine shrimp (*Atremia salina*) and bioassay was prepared by dissolving different salts in distilled water. The composition of artificial sea water is tabulated in table

Table 3.7 Composition of artificial sea water (formulated by Dietrich and Kalle, 1963).

S.N.	Name of salts	Amount used(g/L)
1	NaCl	23.50
2	Na_2SO_4	4.00
3	KCl	0.68
4	H_3BO_3	0.026
5	$MgCl_2.6H_2O$	10.78
6	$CaCl_2.2H_2O$	1.47
7	$NaHCO_3$	0.196
8	Na_4EDTA	0.0003

3.7.5.2Hatching of Brine Shrimp

Brine-shrimp (*Artemia salina*) eggs were hatched in artificial sea water. A spatula full brine – shrimp eggs was placed in beaker of 500 mL containing 400 mL of artificial sea water.After incubation for 48 hours under illumination of table lamp at warm room (approximately 30-32 ^oC), the prototropic nauplii were separated from the eggs. Highly active nauplii of about 1 mm length were collected and immediately used for the bioassay.

3.7.5.3 Sample preparation for bioassay

Solution A was prepared in a 5 mL volumetric flask by dissolving 0.05 g of the each samples (methanol and hexane extract) in methanol (5 mL) and hexane (5 mL) respectively. Solution B was prepared by taking 250 µL from solution A and maintained in 5 mL. Three doses of drugs were used (10,100, & 1000 µg/mL).For each dose level there are 5 replicates of test tubes were used. With the help of the micropipette,100 µL of solution B (for 10 µg/mL)was transferred to 1.25 cm disc of filter paper(Whitman 3) contained in each test tube of 6 ml capacity arranged in five replicates for one dose level. Next 50µl/mL of solution A (for 100 µg/mL) and 500 µL of solution A (for 1000µg/mL) respectively were transferred to separate discs contained in separate test tubes in five replicates. A control test tubes for each dose level prepared using same volume of sample solution used for that dose. All the disc contained in test tubes were dried in air and then evaporated at 37^oC under reduced pressure using a rotatory evaporator by placing the test tubes inside a round bottom flask. The test tubes containing the samples were immediately used for brine-shrimp bioassay.

3.7.5.4 Bioassay with brine- shrimp

Brine- shrimp nauplii can be counted macroscopically in stem of dropper against a lighted background. Ten shrimp were counted using a dropper and transferred into a measuring cylinder that contained a little amount of artificial sea water. The shrimp were transferred into each sample test tube and control tubes of each dose level and volume ofartificial sea water adjusted to 5 mL. There were 5 replicates of sample test tubes and control test tube for each dose level, hence altogether eighteen samples test tubes were prepared for each sample analyzed. These test tubes were kept under illuminated table lamp. After 24 hours, the number of survivors in each sample test tubes were counted by picking out the living shrimp with the help of dropper. Absence of movement of nauplii for five minutes was regarded as dead and percentage death was calculated. The LC_{50} (Lethal Concentration 50) value and 95% CI (Confidence Intervals) were then computed.

CHAPTER 4 -RESULTS

4.1 DNA BARCODING

4.1.1 PCR Amplification and Sequencing

The universal primers specific for four different barcode loci (*matK, rbcL, trnH-psbA*, ITS) (Table 3.5) with specific reaction and cycling conditions (Table 3.7) were used for PCR amplification. All primers pairs successfully amplified the barcode loci under study. On visualization with gel electrophoresis, the approximate band sizes for *matK, rbcL*, *trnH-psbA* and ITSwere found to be 800, 600, 400, and 700 base pairs respectively (Plate 4.4). Sequencing was done by Macrogen Inc., South Korea.

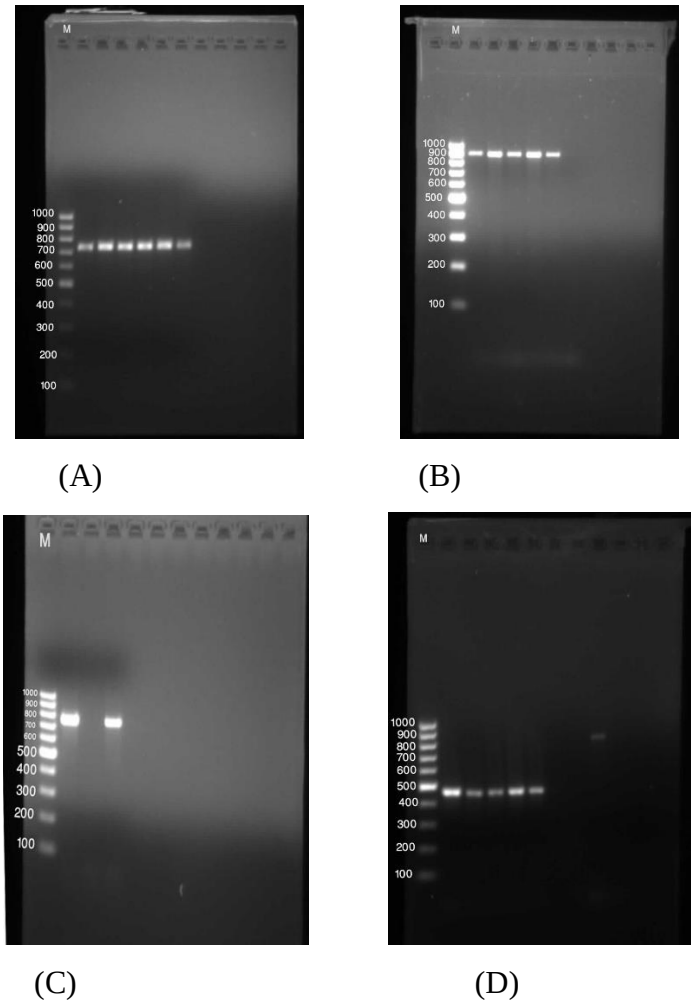


Plate 4.1 DNA bands of amplified barcoderegions. (A) Represents amplified *rbcL* sequences of different spp. (B) represents amplified *matK* sequences; (C) represents amplified ITS sequences and (D) represents amplified *trnH-psbA* sequences. M represents 100 bp+ molecular weight markers.

4.1.2Sequence retrieval and analysis

Sequence data from Macrogen Inc., South Korea was retrieved from Macrogen website and analyzed using CodonCode Aligner v. 4.0.4. Consensus sequences were generated by using CodonCode Aligner software after end trimming and assembling of the reverse and forward sequence of respective Rhododendrons species. Reference sequences retrieved from National Center for Biotechnology Information (NCBI) nucleotide database were used for sequence length editing. ITS sequence of *R.hybrid cultivar (*accession AJ626912.1)was used as reference sequence to edit ITS sequences while for rbcL, sequences *of R.simsii*(accession GQ997829.1) was used. In case of *matK, matK* sequence of *R.hippophaeoids*(Accession HU062135.1),*R.arboreum* (NCBI accession KF521895.1),*R.trichocladum*(NCBI accession AF454856.1),*R.phaeochrysum*(NCBI accession EU087351.1)were used while for *trnH-psbA*, *trnH-psbA* sequence of *R.wardii(*NCBI accession GQ997829.1),*R.sinogrande*(NCBI accession JN046966.1) and *R.tephropeplum*(NCBI accession JX989278.1) were used. *matK*sequence of species *R.barbatum* and *R.hodgsonii, rbcL* sequence of species *R.anthopogon* and *R.lepidotum*, trnH-psbA sequence of *R.barbatum,R.setosum* and *R.arboreum* lacked contig assembly and thus were excluded from phylogenetic study. The total lengths of sequences of various barcode regions after editing are presented in Table 4.2.

Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) was performed with sequences of assembled contigs of all the barcode loci for all accessions to obtain sequences producing significant alignment which is presented in the table 4.1.

Table 4.1BLAST result of query sequences of barcode loci of all accessions with NCBI nucleotide sequence database.

Barcoding loci	**Query Accessions**	**Identity with**	**Total score**	**Query cover**	**E - value**	**Identity**	**NCBI Accession number**
ITS	*R.barbatum, R.arboreum, R.anthopogon, R.stosum, R.campylocarpum R.lepidotum R.nivale R.hodgsonii R.campanulatum*	*R. hybrid cultivar*	1352	99%	0.000	99%	AJ626912.1
matK	*R.anthopogon*	*.hippophaeoids*	2024	97%	0.000	99%	HU62135.1
	R.arboreum	*R.arboreum*	2181	92%	0.000	99%	KF521895.1
	R.setosum R.lepidotum	*R.trichocladum*	1978	93%	0.000	99%	AF454856.1
	R.campanulatum	*R.phaeochrysum*	1983	99%	0.000	99%	EU087351.1
	R.nivale	*R.trichocladum*	1844	96%	0.000	97%	AF454856.1
rbcL	*R.barbatum, R.arboreum R.setosum R.campylocarpum R.campanulatum R.nivale R.hodgsonii*	*R.simsii*	1221	95%	0.000	99%	GQ997829.1
trnH-psbA	*R.campylocarpum R.capanulatum*	*R.wardii*	619	83%	1e-173	97%	HQ707037.1
	R.hodgsonii	*R.sinogrande*	630	77%	5e-177	98%	JN046966.1
	R.anthopogon R.lepidotum R.nivale	*R.tephropeplum*	726	92%	0.000	97%	JX989278.1

4.1.3Characteristics of the four Barcode loci

Of the total nine samples selected for study, the PCR success rate for all the barcode loci were 100% while the sequencing efficiency was 100% for ITS, 77.80% for *rbcL* and *matk* while 66.67% for *trnH-psbA*. The aligned sequence lengths for four barcode regions were 872, 746,511 and 876 bps for *matK, rbcL, trnH-psbA*and ITS respectively (Table 4.2).

Table 4.2 Evaluation of four DNA markers using Clustal W.

Parameters	*matK*	*rbcL*	*trnH-psbA*	ITS
Percent success of PCR	100%	100%	100%	100%
Percent success of sequencing	77.80%	77.80%	66.67%	100%
Aligned length (bp)	872	746	511	876
No. of variable sites	435	172	278	533
No. of parsimoniously informative sites	124	133	228	119
No. of samples	7	7	6	9

4.2 Phylogenetic Analysis

4.2.1 Selection of best-fit model of nucleotide substitution

jModelTest v. 2.1.4 (Darriba *et al.*, 2012) was used to determine the best fit model for nucleotide substitutions for the different accessions used in the study using Bayesian information criterion (Table 4.3).

Table 4.3 Best fit model of nucleotide substitution as determined using jModelTest.

Locus	*matK*	*rbcL*	*trnH-psbA*	ITS
Best Fit Model	HKY+G	HKY+I	TPM1uf	K80+G
BIC value	13175.0687	6651.6830	4444.2919	6593.4435

WhereTPM1uf = Kimura 3-parameter (K81) with unequal base frequencies (uf), +I = invariable sites; +G = Gamma Distribution, HKY= Hasegawa; Kishino and Yano, K80= Kimura, 1980

Lower BIC values signify the best model among all others. jModelTest based analysis showed the lowest BIC score of 6651.6830 for HKY+I Model for *rbcL*sequence. Similarly,best fit model for matK sequence was HKY+G with BIC score of for *matK* 13175.0687. Similarly, for*trnH-psbA* sequence, TPM1uf model showed lowest score of 4444.2919 and hence it was found to be the best fit model. For ITS sequence, K80+G model with score value of 6593.4435 was found to be the best fit model of nucleotide substitution.

4.2.2 Phylogenetic Analysis

Phylogenetic trees were constructed to depict the evolutionary relationship between different species of Rhododendrons. Using jModelTest (v. 2.1.4), a model based phylogenetic tree was constructed and using MEGA (v. 6.0) a maximum parsimony (MP) tree was constructed for different barcode loci. Using best fit nucleotide substitution model from jModelTest (v. 2.1.4), strict consensus trees were generated with confidence interval of 95%. Two outgroup of *Gaultheria* spp. were selected for phylogenetic tree construction.

4.2.2.1 Phylogenetic analysis using*matK* sequences

The strict consensus tree for *matK* sequences based on HKY+G model showed *Rhododendrons species* in a single clade separated from outgroup sequences and from reference sequence at branch length of 23 demonstrating that 23 nucleotide changes were necessary to separate the Rhododendron species from outgroup sequences and reference sequencesfrom NCBI (Figure 4.1). All the *Rhododendron spp* under this study were shown to be sister species to one another. *R. arboreum*was shown the sister species of *R.campylocarpum* at branch length 15 demonstrating that 15 nucleotide changes were necessary to separate this clade. Similarly, with 18 subsitution, *R. anthopogon, R. setosum*& *R. campanulatum*, *R. arboreum* and *R. campylocarpum* clades were separated.

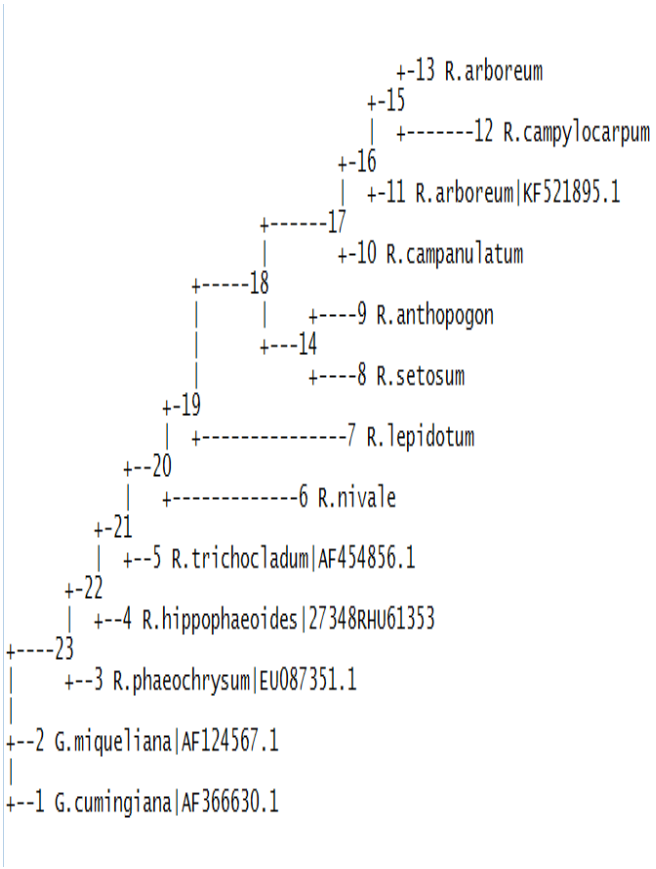


Figure 4.1 A strict consensus phylogenetic tree for ***matK* sequences** using HKY+G model under corrected Bayesian information criterion (BIC). Branch lengths are the expected number of substitutions per site.

Similarly, using MEGA v.6.0 software, a phylogenetic tree based on Maximum Parsimony with 1000 bootstrap replicates was also constructed. *matK* data set generated maximum parsimonious trees with Consistency Index (CI) value 1.000 and Retention Index (RI) value 1.000. The tree was less resolved because branches corresponding to partitions reproduced in less than 50% trees were collapsed (Figure 4.2).

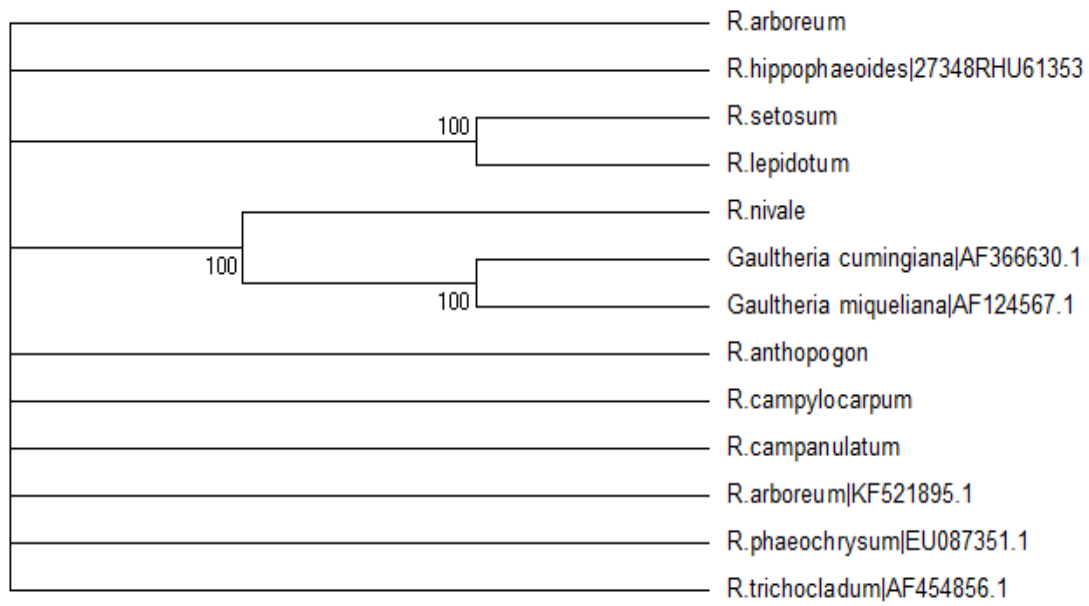


Figure 4.2 Phylogenetic tree for ***matK* sequence** to depict evolutionary relationship between different species *of Rhododendrons* and outgroup sequences retrieved from NCBI nucleotide database with GeneBank accession numbers within parentheses. Numbers at the branches indicate the frequency of occurrence of that branch in all trees (bootstrap support).

Comparison of branching patterns of different sequences between phylogenetic trees generated by jModelTest and MEGA revealed similar branching pattern thereby confirming the validity of phylogenetic analysis. *matK* sequences were able to distinguish the Rhododendrons sequences from outgroup sequences and placed all the accessions in single clade. In the MP tree, outgroup species formed separate clade and *R. nivale* was shown sister species to them. Other Rhododendron spp. formed ploytomy in the cladogram.

4.2.2.2 Phylogenetic analysis using *rbcL* sequences

rbcL based strict consensus tree using HKY+I model showed that all the accessions along with outgroup sequences of *Gaultheria dumicola and Gaultheria heteromera* were grouped into a single clade with a branch length of 12 indicatedthat 12 nucleotide changes were necessary to separated accession of nine Rhododendron spp. from two outgroup sequences. This showed that based on*rbcL*all Rhododendron spp. under study formed polytomy and were not further differentiated into specific sub-clade. NCBI accession *R. simsii* is shown to be sister species of two outgroup accsessions.

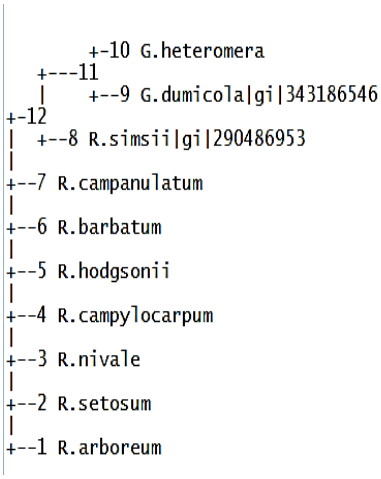


Figure 4.3 A strict consensus phylogenetic tree for ***rbcL*** **sequences** using HKY+I model under corrected Bayesian information criterion (BIC). Branch lengths are the expected number of substitutions per site.

Similarly, maximum parsimony trees were generated using *rbcL* sequences with 1000 bootstrap replicates in MEGA v 6.0 (Figure 4.4). All species and two outlier sequences (*Gaultheria dumicola and Gaultheria heteomera*) were placed in a single clade similar to that of strict consensus tree generated using jModelTest thus indicated the validity of result obtained. The consistency index value for *rbcL* based MP tree was 1.000. The tree was more resolved in comparison to model based tree and two trees were more or less congruent. In the MP tree*R. nivale* and *R. setosum* formed a monophyletic clade and were shown sister species to outgroup spp. and *R. simsii*(Figure 4.4).

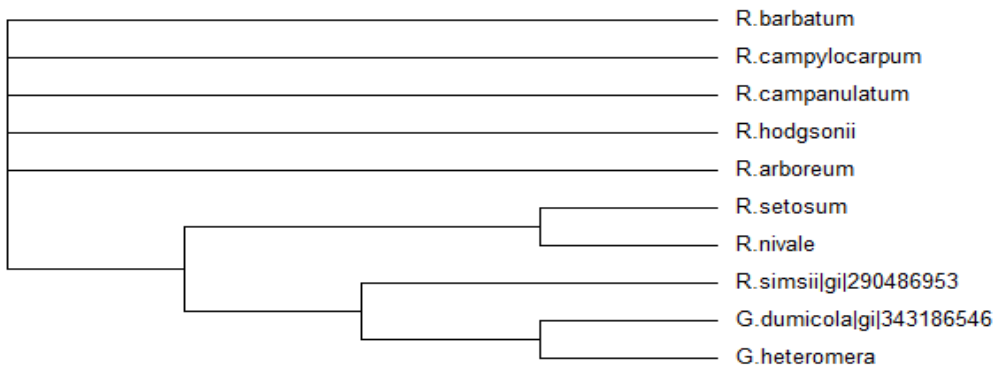


Figure 4.4MP phylogenetic treegenerated for ***rbcL*** **sequence** to depict evolutionary relationship Rhododendrons species and reference sequences retrieved from NCBI nucleotide database with GeneBank accession numbers within parentheses.

4.2.2.3 Phylogenetic analysis using*trnH-psbA* sequences

In case of phylogenetic analysis using *trnH-psbA* sequences of *Rhododendron spp TPM1uf*, a strict consensus tree using TPM1uf model showed accessions into a single clade at branch length of 18 (Figure 4.5).The two main clades were visible in the cladogram. On the first clade, *R. lepidotum* and *R. nivale* shown sister species at branch length 17 demonstrating that 17 nucleotide changes were necessary to separate these species. Similarly, on the second clade accession *R. anthopogon* shown the sister species with *R. tephropeplum* (reference sequence from NCBI) at branch length 12 demonstrating that 12 nucleotide changes were necessary to separate these species.*R. capylocarpum* and R. wardii (reference sequence from NCBI) shown the sister species at branch length 14, *R. campylocarpum* and *R. hodgsonii* at 15, *R. hodgsonii* and *R.campanulatum* at 16 demonstrated that these number of nucleotides changes were necessary to separate these species.

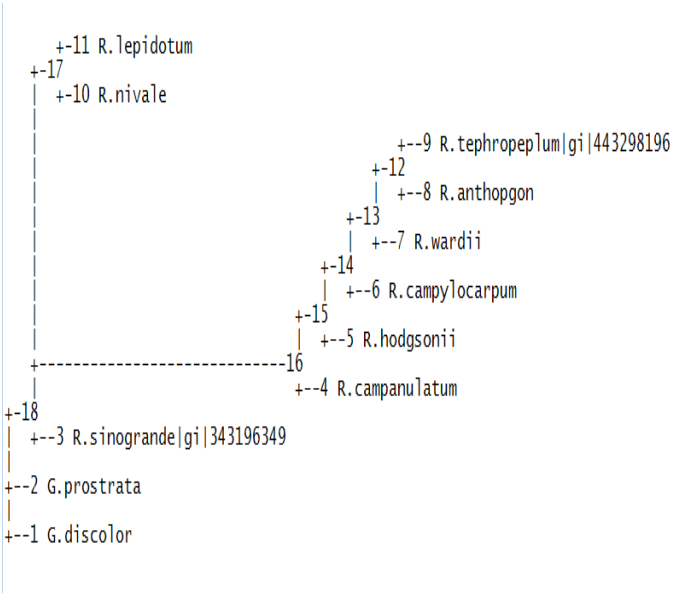


Figure 4.5 A strict consensus phylogenetic tree for ***trnH-psbA* sequences** using TPM1uf model under corrected Bayesian information criterion (BIC). Branch lengths are the expected number of substitutions per site.

Similarly, maximum parsimony trees were generated using *rbcL* sequences with 1000 bootstrap replicates in MEGA v 6.0 (Figure 4.6). All accession species and two outgroup sequences (*Gaultheria discolor and Gaultheria prostrata*) were placed in two clade and shown the same sister species pattern similar to that of strict consensus tree generated using jModelTest thus indicated the validity of result obtained. Thus, jmodel and MP shown the

highly congruent cladogram. The CI value for *rbcL* based MP tree was 1.000. The tree was less resolved because branches corresponding to partitions reproduced in less than 50% trees were collapsed (Figure 4.6).

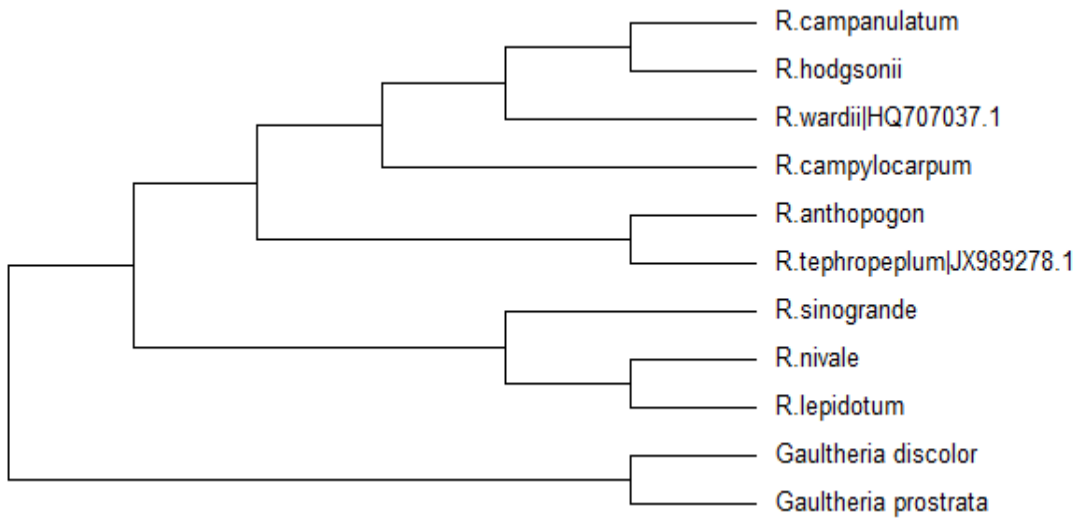


Figure 4.6 Phylogenetic tree for ***trnH-psbA* sequence** to depict evolutionary relationship between *rhododendron species* and reference sequences retrieved from NCBI nucleotide database with GeneBank accession numbers within parentheses. Numbers at the branches indicate the frequency of occurrence of that branch in all trees (bootstrap support).

4.2.2.4 Phylogenetic tree analysis using ITS sequences

In case of ITS sequence, the strict consensus tree constructed using K80+G model showed that the all accession sequences and *R. hybrid cultivar* (reference sequence fromNCBI) visible in the same clade demonstrated that these species are sister species separated from outgroup sequences of *Gaultheriadumicola* and *Gaultheria semi-infera* at branch length of 15 demonstrated that 15 nucleotide changes were necessary to separate the accession from the outgroup (Figure 4.7). *R. setosum* and *R.anthpogon* sown the sister species at branch length 13, *R. nivale* and *R. lepidotum* at 14,*R.campanulatum*, *R. barbatum*, *R. hodgsonii*, *R. campylocarpum* and *R. hybrid cultivar* (reference sequence from NCBI) placed in the same clade which are different from outgroup by 15 nucleotide changes. *R. arboreum* shown the highest evolutionary distance from all these accessions.

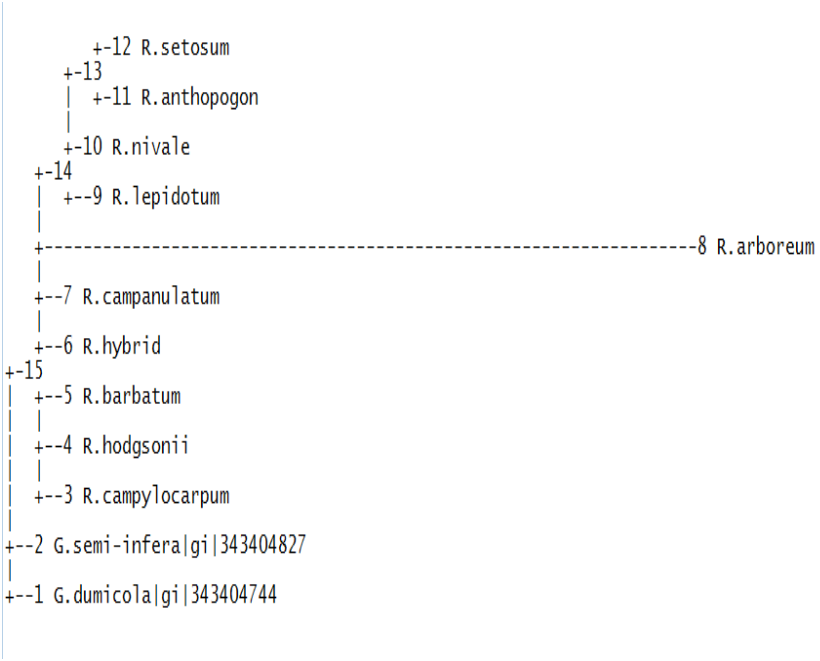


Figure 4.7 A strict consensus phylogenetic tree of **ITS** using **K80+G** model under Bayesian information criterion (BIC). Branch lengths are the expected number of substitutions per site.

The same result was observed with Maximum Parsimonious Tree using ITS sequences with 100 bootstrap replicates (Figure 4.8). The CI value for MP tree was 0.951 and Retention Index value is 0.982. Based on ITS sequences, maximum parsimony tree shown the *R. lepidotum, R. anthopogon, R. setosum and R. nivale* are sister species which formed a monophyletic clade. Similarly, *R. hodgsonii, R. campanulatum, R. campylocarpum, R. barbatum* and *R. hybrid cultivar* (reference sequence from NCBI) shown the sister species and hence visible in the single clade but *R. arboreum* shown the greater evolutionary distances from other species. In jModel tree all Rhododendron shown to be monophyletic while MP tree is shown to be paraphyletic on MP tree. *Rhododendron arboreum* shown to be sister species of outgroup. Monophyletic clade consisting *R. setosum, R. anthopogon, R. nivale*&*R. lepidotum* are seen in both the cladogram. Hence these jModel shown partially congruent with MP tree.

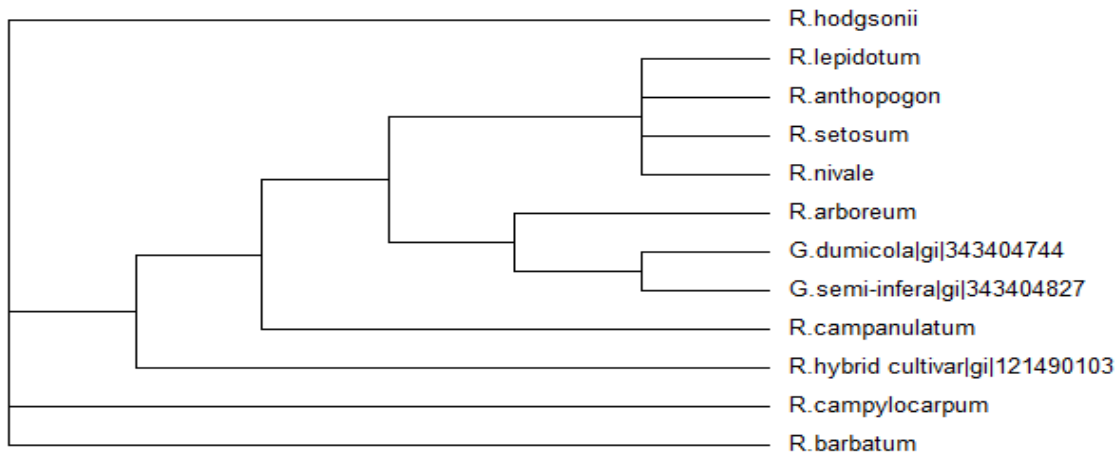


Figure 4.8MP phylogenetic tree generated for **ITS sequence** to depict evolutionary relationship between *Rhododendron species* and reference sequences retrieved from NCBI nucleotide database with GeneBank accession within parentheses.

4.3Chemical and Biological characterization of *Ephedra gerardiana* extract

4.3.1Yield of methanolic and Hexanolic extract of rhizome of *Ephedra gerardiana*

Hexane and Methanolic extract of *Ephedra gerardiana* was used for the phytochemical screening, antibacterial as well as for bioassay analysis. The methanolicextraction yielded higher amount of extract as compared to hexane extraction of plants are listed in Table 4.5.

Table 4.4 Percentage yield of *Ephedra gerardiana* extract in methanol and hexane.

S.N.	Solvent	Wt. of powder (gm)	Wt. of Extract (gm)	% yield
1.	Methanol	100	23.97	23.97
2.	Hexane		9.94	9.94

4.3.2Phytochemical screening

Different phytochemical constituents presents in the methanol and hexane extracts were identified by the colour reactions by using different reagents following the standard phytochemical screening methods (Harborne, 1973, Trease and Evans 1989 and Sofowora, 1993).The main objective of the phytochemical screening was to identify the main groups of chemicals present in methanol and hexane extracts. Results of Phytochemical screening are summarized in Table 4.6.

Table 4.5 Phytochemical screening of the extracts of *Ephedra gerardiana.*

S.N.	Family of natural constituents	Name of screening test	Mathnolic extract	Hexane extract
1	Alkaloids	(a) Mayer's test	-	-
		(b) Wagner test	-	-
		(c)Dragendroff test	-	-
		(d)Hager test	-	-
2	Carbohydrate	(a) Molisch test	+	+
		(b) Reducing sugar test	+	+
3	Flavonoids	a) Lead acetate test	+	+
		b) Shinoda test	+	-
		c) Shibata test	+	-
4	Lactones	Baljel test	+	+
5	Glycosides	Alkaline regent test	+	+
6	Oxalates	Oxalates test	-	-
7	Quinones	Quinones test	+	-
8	Steroids	Salkowski test	+	+
9	Terpenoids	Tschugajen test	+	+
10	Tannins andpolyphenols	Braymer test	+	-
11	Proteins & amino acid	Xanthoproteic test	+	+
12	Saponins	Froth test	+	+
13	Fatty Acids	Spot test	-	-
14	Anthraquinones	Borntiager test	-	-
15	Leucoanthocyanins	Leucoanthocyanins	-	+
		anthracenosides		+

Note: + = Present; - =Absent

4.3.3Antibacterial Susceptibility Assay

All of the seven bacterial strains under this study shown no visible Zone of Inhibition (ZOI) whereas Gentamycin used as positive control shown Zone of Inhibition. This result indicated that bacterial strains are resistant to plant extract presented in table 4.7

Table 4.6 Antibacterial susceptibility assay of *Ephedra gerardiana*

S.N	Pathogenic bacteria used	Zone of Inhibition by methanol and hexane extract		Positive control (Gentamycin disc)	Remarks
		Methanol	Hexane		
1	*Escherichia coli*	_	_	+	No Visible Zone of inhibition (ZOI) in either of the extract observed.
2	*Klebsiella pnemoniae*	_	_	+	
3	*Pseudomonas aueruginosa*	_	_	+	
4	*Salmonella typhimurium*	_	_	+	
5	*Bacillus subtilis*	_	_	+	
6	*Staphylococcus aureus*	_	_	+	
7	*Micrococcus luteus*	_	_	+	

Note: + =Zone of inhibition, **-** = No Zone of inhibition

4.3.4Brine-Shrimp Bioassay

The brine –shrimp bioassay was performed according to the procedure described in section 3.8.4. The extract obtained after complete evaporation of the solvent from the methanol and hexane extracts of the leaf of *Ephedra gerardiana* were bioassayed separately using newly hatched brine-shrimp nauplii. After 24 hours, the number of nauplii survived and died were counted and the results are obtained presented in Table 4.8. All the naupalii remained active with no mortality in the control.

Table 4.7 Results of Brine – Shrimp bioassay after 24 hours count.

Samples	Replicates	Dose level					
		10µg/mL		100µg/mL		1000µg/mL	
		Survivor	Death	Survivor	Death	Survivor	Death
Methanol extract	Control	10	0	10	0	10	0
	1	6	4	3	7	0	10
	2	7	3	4	6	0	10
	3	8	2	4	6	0	10
	4	8	2	5	5	0	10
	5	6	4	5	5	0	10
Hexane extract	Control	10	0	10	0	10	0
	1	6	4	4	6	3	7
	2	7	3	5	5	4	6
	3	8	2	6	4	4	6
	4	6	4	5	5	2	8
	5	9	1	5	5	3	7

The number of survived naupalii decreased as the concentration of drugs increased in both hexane and methanolic extracts but methanolic extract was shown more effective to kill naupalii than the hexane extract as presented in Table. 4.7. Methanolic extract at 1000 µg/mL concentration killed 100 % of the naupalii.

Regression calculations for the assays using different samples are presented in Table 4.8.

Table 4.8 Regression calculation for the Brine-shrimp bioassay to evaluate cytotoxic activity of the plant extracts and compounds

Samples used	n	x	y	xy	X^2	∑x	∑y	∑xy	∑X^2	β	α	X
Methanol extract	10 100 1000	1 2 3	3 5.8 10	3 11.6 30	1 4 9	6	18.8	446	14	3.7	-1.13	1.66
Hexane extract	10 100 1000	1 2 3	2.8 5 10	2.8 10 20.4	1 4 9	6	14.6	33.2	14	2.0	0.87	2.07

Table 4.9 Brine –shrimp cytotoxicity assay of *Ephedra gerardiana*

Samples	**Percentage death at 24 hour/Dose**			**LC_{50} (µg/mL)**	**95% Confidence Interval (µg/mL)**	**Results**
	10 (µg/mL)	**100 (µg/mL)**	**1000 (µg/mL)**			
Methanolic extract	30	58	100	45.71	46.36-45.06	Toxic
Hexane extract	28	50	68	117.49	118.14-116.84	Toxic

The extracts studied in this work showed significant lethality against brine shrimp, which has been successfully used as a simple biological test to guide the fractionation process of plant extracts in order to detect antitumour compounds. This bioassay has good correlation with the human solid tumour cell lines. LC_{50} values < 1000 µg/mL are considered significant for crude extracts.Lower the LC_{50} value higher the toxicity.

The LC_{50} results of the hexane and methanolic extract evaluated in this screening are listed in Table 4.10. The methanolic extract of the species from plant *E. gerardiana* were most active than the hexane extract. These extracts can be regarded as a promising candidate for plant –

derived antitumor compounds. In fact, from these two extracts, methanolic extract (LC_{50} =46.36 – 45.06 µg/mL) was shown to have higher cytotoxic effect than hexane extract (LC_{50} = 118.14 – 116.84) as confirmed by this bioassay.

CHAPTER 5 - DISCUSSIONS

Discussions

The present study was conducted to st [A barcoding and phylogenetic analysis of *Rhododendron spp* samples from Sagarmatha National Park (SNP) using the DNA sequences obtained using the PCR- based DNA sequencing molecular markers. Furthermore, the phytochemical screening, the antibacterial activity and brine- Shrimp bioassay of hexane and methanolic extract of *Ephedra gerardiana* were assessed.

Nepal with diverse geo-climatic conditions, is a storehouse of various very high MAPs. It is reported that approximately 1950 species of high medicinal value plants are found in Nepal (Ghimire *et al.*, 2008). Various species of plants found in Nepal, such as *Neopicrorhiza scrophulariiflora* (Pennel) Hong, various *Rhododendron spp*, *Swertia chirayita* (Roxb. Ex. Fleming), *Podophylum hexandrum* Royle are highly demanded species that are collected and traded to fulfill national and international demand (Edwards, 1996). Every year an average of 20,000 tons of raw medicinal plants, worth between 8.6 to 27 million US dollars is exported from Nepal to many countries of the world including India, China, Europe, the US etc (Edwards, 1996, Subedi, 1997).

This growing demand of Nepalese MAPs has ultimately put pressure on these resources in wild and due to various anthropogenic activities including land fragmentation, degradation, over exploitation and climate change. Therefore these highly valuable bioresources of Nepal have to be judiciously managed and conserved. This has necessitated research and developmentsactivities in multidisciplinary areas including taxonomy, ecology, molecular, chemical, conservation biology, biotechnology etc.

In the present study, an attempt has been made to generate DNA barcode sequences of nine of the Rhododendron spp found in SNP region of Nepal. The generated barcodesequences of four loci of all spp.were also employed in the study of phylogenetic relationship (evolutionary relationship).

Once the generated sequences are submitted to NCBI, they will enrich gene bank and will be future references to all who attempt to identify various Rhododendron spp. found in Nepal and elsewhere the world. Present investigation also attempted to characterize *Ephedra gerardiana* collected from SNP region at chemical level, which has furnished valuable insights on phytocytotoxicity of *E. gerardiana* extracts.

5.1 Sequence analysis and characteristics of *matK, rbcL, trnH-psbA* and ITS regions

For PCR successful, DNA barcoding and molecular phylogenetic research, successful PCR amplification and DNA sequencing are inevitable. PCR amplification was successful for all the loci of all the nine species of Rhododendrons included in this study. The bidirectional sequencing was found to be 100% for ITS, 77.80% for *matK*&*rbcL* while 66.67% for *trnH-psbA*.The *trnH-psbA* intergenic spacer posed greater problem in obtaining bidirectional read with few ambiguous bases due to high frequency of mononucleotide repeats which disrupts sequencing reads and *matK* required manual editing and produced fewer bidirectional reads.

The band size for *matK* after amplification was found to be approximately 900 base pairs for all the species. Similarly, band size for *rbcL* and ITS was found to be 750 base pairs. Band size for *trnH-psbA* was found to be approximately 450 base pairs (Table 4.2). The amplicon length range in base pairs for *matK, rbcL, trnH-psbA* and ITS has been reported in the range of 862-910 bps, 654-654 bps, 226-934 bps and 407-1630 bps respectively and the median amplicon length in bases in completely sequenced plastid genomes was found to be 880, 654, 500 and 705 bps respectively (Hollingsworth *et al.*, 2011).

Using BLAST (Altschul *et al.*, 1990) found in the NCBI web interface a sequence similarity search was performed. The pairwise sequence matches were ranked by statistical significance. The significance scores help to distinguish evolutionary related sequences from unrelated ones (Xiong, 2006). In BLAST searches, the statistical indicator, *E*-value (expectation value) indicates the probability that the resulting alignment from a database search are caused by random chances. If $E < 1e\text{-}50$, it can be said with extremely high confidence that the database match is result of homologous relationship; if $0.01 < E < 1e\text{-}50$ then the match can be considered a result of homology and if $0.01 < E < 10$ then the match is not considered significant but may indicate distant homology (Xiong, 2006).

Our study might be the first study based on DNA barcoding of *Rhododendron spp.* of Nepalsince no *matK, rbcL, trnH-psbA* and ITS sequences of query sequences were found deposited in NCBI database.

Pairwise and multiple sequence alignment were performed using the four barcode regions among the species and using the outgroup sequences using Clustal W (Thompson *et al.*, 1994) module of MEGA v. 6 (Tamura *et al.*, 2011).The loci *trnH-psbA, matk, rbcl and ITS* was found to have 278, 435, 172 and 533 variable sites respectively (Table 4.2).

5.2 Phylogenetic Analysis

Phylogenetics is the study of evolutionary history of living organisms using tree-like diagrams to represent pedigrees of the organisms (Xiong, 2006). Evolutionary history using fossil records and morphological information has been surpassed by the use of genes and other biological macromolecules to study evolutionary relationship. In phylogenetic studies, a group of taxa descending from single common ancestor is called clade or monophyletic group while group of taxa that share more than one closest common ancestor are referred to as paraphyletic group (Xiong, 2006). Phylogenetic study can be performed using variety of methods, which can be categorized broadly into two viz. character based method and distance based method. Distance-based methods such as UPGMA convert aligned sequences or comparisons between gel-banding patterns (e.g., RAPDs, ISSRs, AFLPs) into pairwise distance matrix and then this matricx is used in tree building method (Lowe *et al.*, 2004). In contrast, discrete methods consider each character as an independent source of information (e.g., maximum parsimony and maximum likelihood).

All phylogenetic methods are based on assumptions about the process of DNA substitution. As a result, all phylogenetic inferences depend on their underlying substitution models. In order to draw inferences properly it is necessary to have confidence in substitution models (Goldman, 1993). Use of one or other nucleotide substitution model affects stages of phylogenetic inferences like estimates of phylogeny, substitution rates, bootstrap values, posterior probabilities or tests of the molecular clock (Posada and Buckley, 2004). When the model of evolution assumed is wrong, the phylogenetic methods become less accurate and inconsistent (Bruno and Halpern). Hence, in order to accurately predict the phylogenetic analysis, appropriate models should be determined and used.

The best fit model of nucleotide substitution were selected for each of the four loci viz. ITS, *matK, rbcL* and *trnH-psbA* the barcode loci was determined on the basis of using the software jModelTest v. 2.1.4 (Darriba *et al.*, 2012) using BIC, technically is an asymptotically unbiased estimator of the expected relative Kullback-Leibler information quantity or distance (K-L) (Kullback and Leibler, 1951) which represents the amount of information lost when using a model to approximate another model. For *trnH-psbA* sequence, the best nucleotide substitution model was found to be TPM1uf(Posada, 2003) (Tranversion Model with invariable sites) with the lowest BIC score of 4444.2919. TPM1uf is also a Kimura 3-parameter (K81) with unequal base frequencies. It assumes unequal base frequencies with substitution rates between nucleotides as AC=GT, AT=CG and AG=CT. Transversion model assumes that there are variable base frequencies with variable transversion rates but equal transition rates (AC, AT, CG, GT, AG=CT). For *rbcL* sequence, with the lowest BIC score of 6651.6830, HKY+I (Kimura, 1981)) was found to be the best substitution model. This model assumes unequal base frequencies with substitution rates as AC=AT, CG=GT and AG=CT. For ITS, the best nucleotide substitution model was K80+G with the lowest BIC score of 2892.29. For *matK*, the best nucleotide substitution model was HKY+G with lowest BIC value of 13175.0687. Common models of nucleotide substitution include parameters that describe base frequencies, the substitution rates among the four nucleotides or the distribution of the rate of evolution among sites (rate variation) and among lineages (the molecular clock) (Posada, 2003).

Based on the models, a phylogenetic strict consensus tree for each locus was created with a confidence interval of 95% using jModelTest v. 2.1.4. These trees were compared with Maximum Parsimony trees constructed for all the respective barcode loci. All model based trees were congruent with their respective parsimonious trees.

5.2.1Phylogeneny Analysis using Maximum Parsimony

By using MEGA v. 6.0, Maximum Parsimonious (MP) trees for each locus was constructed and Consistency Index (CI) and Retention Index (RI) of all parsimonious trees were also computed. The value of CI for*matK*and *rbcL* was found to be 1.000 and RI for both found to be 1.000 while CI for *trnH-psbA* was found to be 0.951 and RI 0.982. Similarly, for ITS CI found to be 0.984 and RI found to be 0.95. CI value indicates the realness of clades present in generated cladograms (Shrestha, 2001). CI is widely used method to measure homoplasy which corresponds to the minimum amount of possible evolutionary change divided by actual

tree length (i.e. the number of actual genetic changes in the tree). Lower values of consistency indices indicate that many characters are contradictory to the evolutionary tree. Maximum value of CI means no homoplasy. Consistency Index (CI) for both *matK* and *rbcL* was found to be maximum, 1.000 means no homoplasy. It means these spp does not shared the common characters and evolved from common ancestors. When a maximum parsimony tree is generated, the given set of data may yield a set of very similar trees with same tree length and generating a consensus tree may be useful to represent common features of this set of trees (Rohlf, 1982).

In phylogeny, the relations between taxa are presented in the form of tree with recent taxa at terminal ends. Such arrangement can be of three types: monophyly, paraphyly and polyphyly. Monophyletic groups contain taxa descended from single common ancestor such that two taxa share a unique common ancestor not shared by any other taxa (Xiong, 2006). A paraphyletic group meanwhile contains taxa that share more than one closest common ancestors (Xiong, 2006). In a polyphyletic group, however, some lineages of a population join with some lineages of second population to form a clade. In interspecific phylogeny, polyphyletic groups are considered to be based on convergent characters while paraphyletic groups are considered to be based upon shared primitive characters (Lowe *et al.*, 2004).

Phylogenetic trees are evaluated by some estimate of confidence interval, more commonly using bootstrap method (Felsenstein, 1985). The bootstrap method is a non-parametric statistical analysis which is used to assess the confidence limits on phylogenics. It tests the monophyly of individual clades (Sanderson, 1989). In this method, a pseudoreplicate sample is generated with sample size similar to original data. Since, the sampling occurs with replacement it is likely that some characters appear more than once in the pseudoreplicate and some characters may not be represented at all. The newly generated data matrix is used to generate a tree and the process is iterated as many as 100-1000 times.

Comparing model averaged phylogeny and maximum parsimony trees, *matK* sequences of all *Rhododendron spp* and outgroup sequences of *Gaultheria cumingiana* and *Gaultheria miqueliana*, although formed monophyletic clade, *R. setosum* and *R. lepidotum* formed subclade inside the large clade indicated these two species diverged from other species (Figure 4.1 and 4.2).In case of *rbcL* sequences, all the accessions and outgroup sequences of *Gaultheria dumicola and Gaultheria heteromera* appeared as a single clade but *R. setosum* and *R. nivale* formed subclade inside the large clade indicating these spp diverged from other spp. (Figure 4.3 and 4.4). Based on *trnH-psbA* sequences, although all accessions under study

formed a single monophyletic clade from single ancestor *Gaultheria spp*, but formed some subclade inside the main clade. *R. nivale* and *lepidotum* formed subclade, indicating these spp diverged from other spp. and close to the reference sequence of *R. sinogrande. R. anthopogon* formed subclade with reference sequence *R. tephropeplum.* Similarly, *R. campanulatum* fromed subclade with *R. hodgsonii* indicatedthese spp. diverged from other spp (Figure 4.5 and 4.6).In case of ITS sequence, it was observed that all *Rhododendron spp* and the reference sequence of *Rhododendron spp* from nucleotide database a single monophyletic clade while *R. hybrid cultivar* shown as sister species of other *Rhododendron spp* (Figure 4.7 and 4.8). Incorporation of these sequences in future studies can shed some light on whether *matK,rbcL, trnH-psBA* and ITS can be used as a barcode for identification of *Rhododendron spp* or not, but from the current data it's highly improbable that *matK, rbcL, trnH-psbA* and ITS can be used for identification because it couldn't clearly resolve the accessions of *Rhododendron spp* from that of outgroup sequences.From the present study it is concluded that for generation of potential barcode of *Rhododendron spp* identification, needs further research using other barcode loci as well as combination of barcode markers. But this result has to be further verified with samples from all over the country and thus this research has opened a new avenue for further research of *Rhododendron spp* in Nepal.

5.3Phytochemicals screening

Phytochemical constituents such as alkaloids, flavonoids, tannins, phenols, saponins, and several other aromatic compounds are secondary metabolites of plants that serve a defense mechanism against prediction by many microorganisms, insects and other herbivores. The present study carried out on the plant samples revealed the presence of medicinally active constituents. The phytochemical constituents of the selected plants investigated are summarized in Table-4.6. Analysis of methanolic plant extract revealed the presence of carbohydydrate, flavonoids, glycosides, lactones, quinones, terpenoids, steroids and tannins and polyphenols, saponons, proteins and amino acid and absence of alkaloids, oxalates, fatty acids, anthraquinones, leucoanthocyanins. Similarly, analysis of hexane extract revealed the presence of carbohydrate, flavonoids, lactones, steroids, proteins and amino acids, sappnons and leucoanthocyanins and absence of alkaloids, quinones, oxalates, tannins and polyphenols, fatty acids, anthraquinones. Alkaloids, oxalates, fatty acids, anthraquinones were absent in the both plant extracts studied.

Tannins bind to proline rich proteins and interfere with the protein synthesis.Flavonoids arehydroxylated phenolic substance known to be synthesized by plants in response to microbial infection and it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. Coumarins are also known act against gram positive bacteria and it is produced in carrots in response to fungal infection which could be attributed to its antimicrobial activity. Antimicrobial property of saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell. Steroids have been reported to have antibacterial properties, the correlation between membrane lipids and sensitivity for steroidal compound indicates the mechanism in which steroids specifically associate with membrane lipid and exerts its action by causing leakages from liposomes.

5.4Antibacterial Susceptibility Assay

Infectious disease caused by bacteria, viruses, fungi and parasites are still a major threat to public health (Cos *et al.*, 2006). In the past three decades, a lot of microbial resistance to antibiotics have been observed (Chopra *et al.*, 1997). This has led to research on development of new anti-microbial drugs in order to treat the infectious disease and thus the attention has been drawn to medicinal plants in order to identify potential drugs since medicinal plants contain a wide range of substance that can be used to treat lots of infectious diseases with reduced side effects (Duraipandiyan *et al.*, 2006).

In the present study, both methanolic and hexane extract antibacterial assays were carried out but none of the extract possessed the antibacterial activity (Table 4.7).

When the bacteria are resistant to drugs, they are nevertheless able to acquire new mutations called adaptive mutations (Cairns, 1988). There are many mechanism of drug resistance like reduced permeability or uptake, enhanced efflux, enzyme inactivation, alteration or over expression of the drug target and losss of enzymes in drug activation are major mechanism or drug resistance, which are due to the chromosomal mutation acquired due to climatic conditions, over use of the drugs etc. That’s why it is suggest that further research by generating silver nanoparticles and plants extract is necessary to assay the effectiveness of the drugs because antibacterial activity can be enhanced by synthesizing silver nanoparticles from it (Nethradevi *et al.*, 2012)

5.4 Brine- Shrimp Bioassay

Brine shrimp lethality is the simple bioassay useful for screening large number of extracts in the drug discovery process from the Medicinal plants. The procedure of Mayer *et al.,* 1989 was adopted to determine the lethality of plant extracts to brine shrimp. The method allows the use of smaller quantity of the extracts and permits larger number of samples and dilutions within shorter time than using the original test vials.

The degree of lethality was found to be directly proportional to the concentration of the extract. In the evaluation for general toxicity using brine shrimp, maximum mortalities took place at a concentration of 1000 µg/ml whereas; least mortalities were at 10 µg/ml concentration.

Further, the values of percentage death from the 24 hour counts were used to calculate the values of LC_{50} and 95% Confidence interval are presented in table 4.10.The sample displaying LC_{50} values less than 1,000 µg/mL (Mayer et al.,1989) is considered as pharmacologically toxic. In methanol (LC_{50} = 45.71 µg/ml) and hexane (117.49 µg/ml) extract, both extract displayed toxic property but methnalic extract shown more toxic than hexane since the lower the $LC_{50,}$ higher the toxic (Table 4.10).Methanolic extract is more toxic than hexane extract because of presence of both polyphenols and reducing compounds. Although, the Brine – shrimp bioassay alone is insufficient to evaluate the anticancer property of the plants, however, it is reasonably reliable to screen the cytotoxic activity of the plants materials. In this regard, our results clearly indicated that both methanolic and hexane extract bear the potential cytotoxic property and needs further in- depth research on cytotoxic effects of plants extracts on cancerous cell line.Based on the possible relationship between brine shrimp lethality and plant bioactivity, this work could serve for further ethnobotanical and phytochemical research

HAPTER 6:CONCLUSION

6.1 Conclusion

Nepal is endowed with a varied climatic [illegible]ons and abundant ecological habitats, and hence is rich in floral and faunal diversity despite its size. Among the 1950 species of medicinal and aromatic Plants of Nepal, some spp. *of Rhododendron* and *Ephedra gerardiana* are important species in terms of their traditional used and economical values. However, research activities pertinent to their conservation, characterization and sustainable utilization are still scarce. Therefore, present investigation was undertaken with an overall objectives to characterize some *Rhododendron* spp. and *E. gerardiana* at molecular and chemical level to help ongoing conservation and utilization. Nepal Himalyas being one of the climate change susceptible landscapes, prime importance has to be given to valuable MAPs diversity of this region. Besides, over exploitation of high value species to sustain livelihood is another by challenge for Nepal. Present study has generated species- specific DNA barcodes of nine species of Rhododendrons of Nepal using four standard barcode loci. When these sequences will be deposited to NCBI, they will be the primary data foor future DNA – based research in Nepal or elsewhere. So far, many limited sequences of Rhododendron exists in NCBI gene bank. However, in order to develop user friendly PCR –based molecular diagnostics. For the valuable species, further research involving large no. of samples collected from wider geographical regions each of the sample species need to be considered, barcode locus is best for *Rhododendron* spp.identification needs to be investigated. Present study is just a foundation for that bigger study. For such bigger investigations, international collaboration needs to be sought.

Phylogenetic analysis performed using model based and maximum parsimony based methods have furnished different stories regarding phylogeny of nine species under study. *matK* sequences of all *Rhododendron* spp formed monophyletic clade but *R. setosum* and *R. lepidotum* formed subclade inside the large clade indicating these two species diverged from other species. In case of *rbcL* sequences, all the accessions and outgroup sequences appeared in a single clade but due to interspecific variation within the species, *R. setosum* and *R. nivale*

formed subclade inside the large clade indicated these spp diverged from other spp. and shared some gentic characters. Based on *trnH-psbA* sequences, although all accessions under study formed a single monophyletic clade but due to interspecific variation, *R. nivale* and *lepidotum* formed subclade, indicated these spp diverged from other spp. and close to the reference sequence of *R. sinogrande. R. anthopogon* formed subclade with reference sequence *R. tephropeplum.* Similarly, *R. campanulatum* fromed subclade with *R. hodgsonii* indicated these spp. diverged from other spp. and shared some genetic characters by these two spp. In case of ITS sequence, it was observed that all *Rhododendron spp* and the reference sequence of *Rhododendron* spp from nucleotide database a single monophyletic clade while *R. hybrid cultivar* shown as sister species of other *Rhododendron* spp. Therefore, in order to have a complete phylogenetic picture, combined phylogenetic analysis needs to be carried out including more gene loci and more spp.

Incorporation of these sequences in future studies can shed some light on whether*matK, rbcL, trnH-psbA* and ITS can be used as a barcode for identification of *Rhododendron spp* or not, but from the current data it's highly improbable that *matK, rbcL, trnH-psbA* and ITS can be used for identification because it couldn't clearly resolve the accessions of *Rhododendron* spp from that of outgroup sequences. All sequencesgenerated during this study will be deposited in public databases like NCBI or BOLD in near future which will aid in future research for species identification, molecular phylogenography etc. Present DNA Barcoding as well as intrerspecific phylogenetic study on *Rhododendron* sppare novel works performed in Nepal. This study has to be further continued with more species and more number of individual species samples collected from all over the country and thus this research has opened a new avenue for further research of *Rhododendron* sppin Nepal.

Analysis of hexane and methanol plant extract of *E. gerardiana*revealed the presence of vaious phytochemical constituents. It showed more cytotoxic effect of methanol extract than hexane extract to the brine shrimp. So this plants is might be very useful to cure various human ailments including cancer.

6.2 Recommendations

Based on the present study, following recommendations are withdrawn for long term molecular identification, characterization, conservation and sustainable utilization of *Rhododendron* spp and *Ephedra gerardiana* in Nepal.

1) The present study showed that *Rhododendron* spp is highly diverse at the genetic level are collected from conserved region, SNP. Many *Rhododendron* spp. are highly valued medicinal plants found in SNP. Similarly, *E. gerardiana* is another very valued in terms of socio- economy and aesthetic values. Due to their high economic importance, they are being threatening these species to extinction. Therefore, judicious conservation planning and management for their sustainable utilization needs to be performed. In order to generate scientific information relevant to conservationists and forest managing scientific research is various aspects (economical, taxonomical, molecular genetics, climate change, molecular biology and biotechnology need to be conductedin integrated manner.
2) It is recommended that the local people, the harvesters and people practicing traditional medicine are trained and equipped with the knowledge of importance of proper harvesting period to allow outcrossing and pollination so that regeneration of the plant occur in the future.
3) *In situ* conservation strategies, area under high threat should be announced by the government and the seeds and germplasm of these species should be conserved through *ex situ* methods in seed banks and gene banks.
4) From the present study, it is recommended that *matK*, *rbcL*, *trnH-psbA* and ITS can be potential barcode for discrimination of *Rhododendron spp*. For this, it is recommended that further studies taking large species samples and using other barcode loci as well as combination of these loci can identify potential barcode markers for identification of these species found in various parts of Nepal.
5) From the present study, *E. gerardiana* was found to be resistant to the pathogenic bacterial strains, so it is recommended that further studies are necessary to assess the antibacterial activity that can be done by making silver nanoparticles from it. It is also recommended that it shown the toxic effect to the brine – shrimp which is the sign of cytotoxic effect to the cancer cells. So further bioassay on cancer cell lines needs to be undertaken.

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APPENDICES

Appendix I

Preparation of Reagents and Extraction Buffers

1. Preparation of (50X) TAE stock buffer preparation (Tris-Acetate-EDTA)

Tris base (242 gm; Qualigens Fine Chem ımbai) was dissolved in approximately 750 ml deionized water. To this solution, glacial acetic acid (57.1 ml Qualigens Fine Chemicals, Mumbai) was added followed by 0.5 M EDTA (pH 8.0, 100 ml; Promega Corporation, USA). The final volume was made up to 1L. The final solution is (50X) TAE stock buffer. This stock solution can be stored at room temperature (Sambrook and Russell, 2001). The working solution of 1X TAE buffer was made by diluting the stock solution (50X) in deionised water or ddH_2O.

2. Preparation of (5X) Gel Loading Buffer (GLB)

Sucrose (2.5g) was dissolved in deionized water (7ml) in which bromophenol blue (25mg, Fermentas Life Sciences, Canada) was added and the final volume made up to 10ml. This prepared gel loading buffer (GLB) was added to the sample in proportions as 1 (GLB) to 1 (DNA sample) and 1 (GLB) to 4 (PCR product) by volume, during electrophoresis.

3. Preparation of Agarose Gel

Agarose (1.5; Promega, Spain) was dissolved in TAE buffer (100ml, 1X) in the microwave. It was then cooled to approximately 55°C and poured on to the gel casting tray with an appropriate comb (8-17 toothed) fixed in place for well formation.

4. Preparation of Tris Buffer (1M, pH 8.0 and pH 7.5)

Tris Buffer (1M, pH 8.0 and pH 7.5) stock solution was prepared by adding tris base (60.55gm, Qualigens Fine Chemicals, Mumbai) to deionized water (400ml). The pH was adjusted to 8.5 or 7.5 by the addition of concentrated HCL. The final volume was then

made up to 500ml, autoclaved and stored at room temperature until needed for preparation of extraction buffers (Sambrook and Russell, 2001).

5. Preparation of EDTA (0.5M, pH 8.0)

Disodium Ethylene Diamine Tetra acetate.$2H_2O$ (EDTA, 93.05gm, Promega) was added to a Schott bottle containing deionized water (400ml), mixed on magnetic stirrer and the pH was adjusted to 8.0 by adding NaOH pellets (approximately, 10gm). The volume was adjusted to 500ml with deionized water, autoclaved and stored at room temperature until needed.

6. Preparation of NaCl (4M)

Sodium Chloride (NaCl, 117gm, Qualigens Fine Chemicals, Mumbai) was added to Schott bottle containing deionized water (400ml), mixed on a magnetic stirrer. The final volume was adjusted up to 500ml with deionized water, autoclaved and stored at room temperature.

7. Preparation of Graham's CTAB (Hexadecyl Trimrthyl Ammonium Bromide) extraction buffer (4% CTAB, 1.4m NaCl, 0.1M EDTA, 0.1M Tris HCl, pH 8.0) (Graham *et al.*, 1994)

For the preparation of CTAB extraction buffer, Tris base (100ml of 1M solution, pH 8.0) was dispensed on a 1L Schott bottle (sterile) and EDTA (200ml of 0.5M solution, pH8.0), NaCl (350ml of 4M solution) and CTAB (40gm, Loba Chemie, India) were mixed and the final volume was made up to 1L with deionized water.

8. Preparation of TE buffer (Tris-EDTA; 10mM Tris HCl, 1mM EDTA) with RNAse

EDTA (1ml of 0.5M stock; pH 8.0) was added to a bottle containing Tris base (5ml of 1M solution) and the final volume made up to 500ml. This was autoclaved and stored at room temperature. RNAse A was added to Tris-EDTA buffer (200µl of 5mg/ml RNAse A) in

200ml TE buffer (to make final concentration of 10µg/ml) in sterile Schott bottle for fresh use.

9. Preparation of Doyle and Doyle extraction buffer (100 mM Tris Hcl, 0.025 M Na-EDTA, 1.4 M Nacl, 4 % CTAB, 2 % polyvinyl pyrrolidone, Doyle and Doyle, 1990)

Tris Buffer (1ml of 1 M stock, p^H 8.0), Na – EDTA (5 mL of 0.5 M stock), NaCl (3.5 ml of 4 M Stock), 0.4 gm CTAB (Loba Chemie, India), PVP (2 ml) were dispensed and mixed in a bottle. The final volume was adjusted to 100 mL with deionized water. Beta-mercaptoethanol (20 mL) was added to freshly prepared buffer inside a fumehood.

10. Preparation of Primers, dNTPs and DNA Dilution

Primers were diluted to required concentration 10µM (the working concentration) using sterile distilled water. Commercially supplied dNTP mix (10mM each, Fermentas Life Sciences) was used for PCR reaction in research investigation. It was stored at -20°C until use. Dilution of genomic DNA to required concentration was carried out by initial estimation of concentration of DNA by using Bio-photometer (Eppendorff, Germany).

11. Basic lead acetate solution

Lead acetate 2.375 g is dissolved in distilled water (50 ml).This solution is boiled with excess of litharge (lead monoxide) and filtered.

12. Benedict's reagent

Anhydrous sodium carbonate (5 gm), sodium citrate (8.63 gm) and copper sulfate pentahydrate (0.865 gm) dissolved in distilled water (50 ml).

13. Dragendroff's reagent

Bismuth nitrate (4 gm.) is dissolved in 5N nitric acid (10 ml) to make solution A. Potassium iodide (13.5 g) is dissolved in distilled water (20 ml0 to make solution B. These solution A and B are mixed together in a 50 ml volumetric flask and then diluted with distilled water up to the mark.

14. Fehling's A

$CuSO_4$ (35 g) is dissolved in distilled water (500 ml). To this added 3 ml of conc. H_2SO_4.

15. Fehling's B

Sodium potassium tartarate (Rochelle salt) (17.3 g) and pellets of NaOH (60 g) are dissolved in distilled water (500 ml).

16. Hager's reagent

It is a saturated picric acid solution.

17. Mayer's reagent

Mercuric chloride (0.679 g) is dissolved in distilled water in a 50 ml volumetric flask. To this solution is added potassium iodide (2.5 g). The scarlet precipitate is dissolved by shaking and then diluted with distilled water up to the mark.

18. Molisch's reagent

α - Napthol (5 g) is dissolved in methanol (50 ml).

19. Neutral ferric chloride solution

Ferric chloride (1 g) is dissolved in distilled water (100 ml). To this added little sodium carbonate with stirring until persistent of a slight turbidity. The mixture is filtered and the colourless filtrate is used.

20. Sodium picrate solution

Picric acid (0.25 g) is dissolved in distilled water (50 ml) and then neutralized with sodium bicarbonate. Dip a strip of Whatman No. 1 paper in the prepared solution and is dried avoiding contamination to prepare sodium picrate paper for cyanogenic glycosides detection.

21. Wagner's reagent

Iodine (1.27 g) and potassium iodide (2 g) are dissolved in distilled water (100 ml).

Appendix II

Praparation of Media and Reagents

1. Preparation of Muller Hilton Agar (MHA)

Distilled water (500 mL) was added to Muller Hinton Agar (19 gm.) taken in 1 liter conical flasks. The content was boiled with continuous shaking and autoclaved at 121 ^{o}C for 15 minutes. The sterile medium was allowed to cool for some time and poured (about 25 mL/plate) properly in the sterile petri dishes (90 mm diameter) aseptically inside a laminar flow. The plates were left at room temperature for solidification.

2. Mc Farland 0.5 barium sulfate turbidity standard:

The standard was prepared by adding 0.5 mL of 0.04 M bariumchloride to 99.5 mL of 0.36 N sulfuric acid (Koneman, 1988). The bacterial inocula were diluted with distilled water until their color matched with 4-6 mL of the Mc Farland turbidity standard.

3. Preparation of Nutrient Broth and Nutrient Agar):

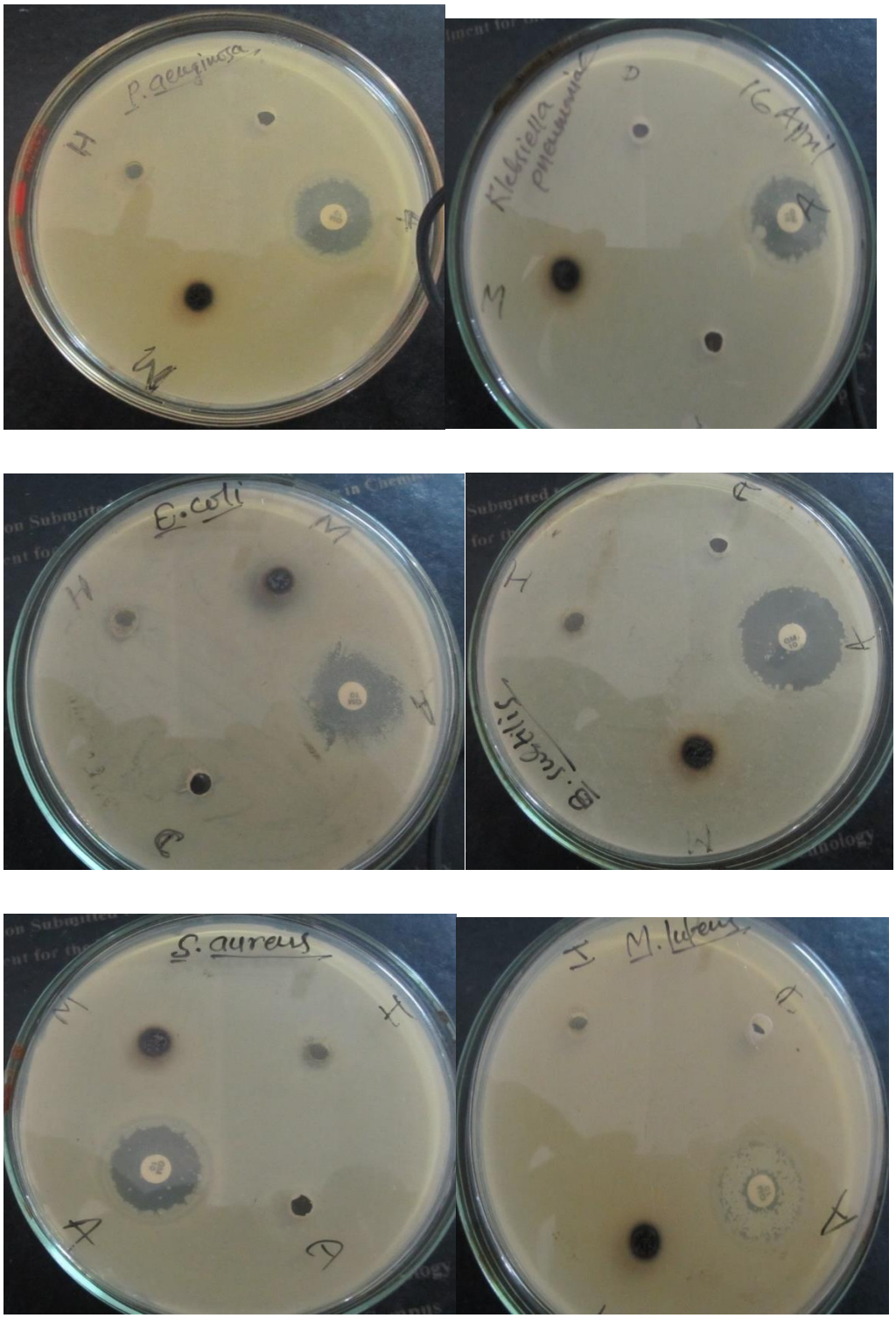
Nutrient broth medium was prepared by dissolving 0.65 g of nutrient broth in 50 mL of distilled water for the growth of bacterial inocula, pH was adjusted to 7.0 and the solution was autoclaved. Nutrient agar medium was prepared by dissolving 2.0 g of nutrient agar in 100 mL of distilled water; pH adjusted to 7.0 and the solution was autoclaved.

Appendix III

DNA Quantification Results obtained using Biophotometer (Eppendorff, Germany)

Sample ID	Dilution Factor	Absorbance (nm)				Ratio 260/280	Ratio 260/230	Concentratio(µg/mL)
		230	260	280	320			
SNP137	10:90	0.056	0.023	0.013	0.000	1.74	0.41	1.1
SNP240	10:90	0.289	0.286	0.158	0.013	1.93	1.02	142.9
SNP269	10:90	0.412	0.595	0.289	0.004	2.06	1.44	297.3
SNP271	10:90	0.269	0.244	0.126	0.015	1.94	0.91	122.2
SNP273	10:90	0.047	0.021	0.002	0.001	1.64	0.44	1.0
SNP283	10:90	0.215	0.089	0.046	0.001	1.91	0.41	4.4
SNP318	10:90	0.580	0.994	0.481	0.019	2.07	1.71	497.0
SNP319	10:90	0.416	0.496	0.258	0.028	1.92	1.19	247.8
SNP323	10:90	0.192	0.081	0.045	0.006	1.80	0.42	40.3

Appendix IV



Note: H denotes hexane extract; M denoted Methanol extract, D denotes DMSO (Negetive control); A denotes Antibiotic disc (Positive control) in plates.

Appendix V

ITS barcode of Rhododendron spp.

R.arboreum

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R.anthopogon

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R.barbatum

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R.campanulatum

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R.campylocarpum

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R.hodgsonii

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R.lepidotum

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R.nivale

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R.setosum

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***MatK* barcode of Rhododendron spp**

R.arboreum

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R.anthopogon

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R.campylocarpum

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R.campanulatum

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R.lepidotum

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R.nivale

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R.setosum

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RbcL barcode of Rhododendron spp.

R.arboreum

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R.barbatum

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R.campylocarpum

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R.campanulatum

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R.hodgsonii

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R.nivale

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R.setosum

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trnH-psbA barcode of Rhododendron spp

R.anthopgon

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R.campylocarpum

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R.campanulatum

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R.hodgsonii

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R.lepidotum

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R.nivale

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