# CHAPTER 1: INTRODUCTION

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### 1.1 Medicinal plants

The medicinal plants and their use in medicinal system has been in main stream since the very early or the ancient period of time and their use may be contemplated as the beginning of the modern medicine period. The Compounds isolated from the medicinally important plants have been considered as important resource of complexes for the drug processing and they are still in use by the pharmaceutical industries to process different drugs. Approximately 10 % which is about 350,000, of all the vascular plants, are considered as medicinal plants. In the very beginning, methods of trial-and-error were used to differentiate the herbal plants having unique or valuable effects on health. The continuity of using these herbal plants has been increased over the time and now this has become the traditional method for the treatment of many diseases. Till today, many cultivars grow different species of plants worldwide for extracting the essential and important components for the drug store or medicine processing industry. Beside this, the authorized definition of the traditional medicinal system can be explained as the total summation of skills, practices and knowledge grounded on the concepts, experiences, beliefs and theories, experiences native to the diverse cultures which can be utilized for maintaining the health along with the treatment, preservation, improvement and diagnosis of physical or mental sickness.[1]

The medicinal or the herbal plants always involved as a crucial part for the basic source of the medication leading components. Ancient humans treat their basic sickness based on their taste, instinct and experience by using herbal plants. Henceforth, longer than the history of humanity is the history of medicinal herbs. The evolution of herbal plants deals with many challenges through the years. Several methods were developed by taxonomists for classifying plants such as anatomic classification, morphologic classification and the chemotaxonomic classification.

Initial approaches are clustered according to the conventional categorization while the last classification is an advanced or modern method of identifying the species of plants.[2]

As far as 18th century, healing and curative properties of plants and their method of treatment, their effect on the humans were known but the active compounds were unknown. Through the beginning of modern genera of science, discoveries of different chemical analysis, chemical separation instruments were made it possible to isolate and distinguish the active compounds associated within the particular plant to treat diseases and disorders. Since then, these active compounds are produced synthetically in the labs to manufacture the medicines further. Today's medicine industries are mainly based upon the secondary metabolites or active compounds of plants, as the processed raw material of many plants are used in drug processing and they are cost effective also.[3]

Most of the plants in polygonaceae family are considered and familiar because of their therapeutic importance treatment and dealing of several disorders and diseases related to humans. The family polygonaceae comprises of shrubs, herbs and rarely trees that includes nearly about 48 genera along with 1200 species. The plants' leaves are mainly stipulate with simple and alternate pattern. Flowers are typically small, actinomorphic and bisexual type. The perianth of flower is mainly comprises of two undifferentiated whorls with 3-6 distinct, basal tepals. The androecium contains 6-8 distinct, basal connate stamens generally in two whorls. The gynoecium involves three carpels, one superior ovary with single locule having basal ovule. Fruit is generally a three-winged achene. The largest genera among the polygonaceae family are *Erigonum* comprises of 240 species, *Rumex* that comprises of about 200 species, *Coccoloba* that comprises 120 species, *Persicaria* having about 100 species and *Calligonum* that comprises nearly of about 80 species. The plants belong to family polygonaceae is present worldwide, but still, they are mostly distributed in the northern temperate zone. In terms of general uses, some species of *Triplaris* provide harvested wood.

Many species are used for common eating purposes even the jelly is made and sold commercially in Florida from the *Coccoloba* species. Buckwheat groats (*Fagopyrum*

species) are very common in eating manner and also are used to make flour. The stalks of rhubarb (*Rheum* species) are mainly eaten in salads or as cooked vegetables, high uptake of rhubarb leaves can be fatal as it contains high amount of oxalic acid. Polygonaceae contains some abundant weeds also such as some species related to *Rumex*, *Persicaria* and *Polygonum* namely Japanese knotweed.[4]

In the world's ranking of mega-biodiversity countries, India exists in the 12$^{th}$ position having very rich flora or vegetation diversity with a widespread range of medicinal plants having a knowledge based on plant's medicinal uses among different ethnic groups of Himalayan areas. Basically, India proves to be in first place among the world's most culturally and biologically diverse country and placed in ninth ranked in the world, in terms of richness in higher flora or vegetation. At the ecosystem level, India is considered as rich with 10 different biogeographic zones.

India also having the two world's biodiversity spots, for the reason of extraordinarily high levels of endemicity, species richness and threatened status. It is assessed that, about 32% of the total India's plants stand now in category of endemic range and found in some specific location and not over all in the world. Among all the species of plants, flowering species plants have much higher fraction of the indigenousness.[5] The Northern Himalayas are flourishing with a wide range of highly demanded medicinal plants. About 30,000 medicinal plant species are there in world in which 8000 species are found in India.[6]

For majority of population in world, the medicinal plants play a vital source as lifesaving drug; India has a plant based ethno-medical tradition. The herbal or medicinal market worldwide is estimated to the cost of US $800 billion a year. In Asia, in the terms of herbal and traditional knowledge systems related to ayurvedic medicines, India is one of the leading nations. Forest plays an important source of income for the people living in both the lowland and mountain areas as the forest provides the diversity of valuable plant products for medicine as well as food. Around 32,600 tons of medicinal plants in a year is exported by India.[7]

This increased the demand of herbal medicine which brings the medicinal plants to a stage of near threatened to the critically endangered. India's northern region alone

flourish with around 1748 species of medicinal plants, most of them (about 1720 plant species) were present at the height of 1800 mtr. On the basis of regions, the many and majorly species of the herbal plants have been reported from the Uttarakhand state, followed by the states like Sikkim and West Bengal. Many medicinally significant plant species stand now in the endemic status to the Indian Himalayan region. From the overall medicinal plant species, around 62 species are now endemic to the Himalayan region whereas the 208 species have extent their distribution to the different areas but they are also close to endemic. Around 200 species of medicinal plants in Himalayan region are consumed in the form of boiled, roasted, cooked or even in raw form, or in the form of pickles or jam, even many of the species are used in the form of spices and oil. Different native groups or communities uses the medicinal plants in direct or indirect form as fodder, food, timber along with the ethano-botanical purposes.[8]

## 1.2 *Rheum* Plant

*Rheum emodi*, locally known by the name rhubarb, is a highly recognised as medicinal herb. It is a sturdy, robust perennial herb which is a member of the polygonaceae family. It has been grown for their therapeutic effects for more than 5000 years. The world's temperate, tropical, and subtropical zones all contain this herb, but Asia is where it is most prevalent and cultivated at a height of about 1800 M. This genus is mainly present in Asian countries like India (Kashmir to Assam), Nepal, China, Bhutan, Pakistan, Tibet, Korea, and Russia. This medicinal herb covers the wide range of medicinal properties specifically antimicrobial, anticancerous, antifungal, antioxidant, hepatoprotective, wound healing, purgative, laxative, stomachic, immune enhancing activity, this all gave the term “wondrous drug” to this world wide used medicinal plant.

*Rheum* contains a variety of compounds such as anthraquinones, an important member in quinone family, which constitutes a variety of compounds such as emodin, aloe-emodin, emodin glycoside, rhein, chrysophanol, physcion with their glycosides,

stilbene which includes resveratrol, picetannol, volatile oil, saponins and tannins. Rhubarb roots powder has a natural healer against wounds.

Almost all parts of *Rheum* species are utilized in Chinese and Indian medicine system, as its rhizomes preparation are extensively used in the treatment of wounds, cuts, and bowel like complaints. This herb is frequently used in several old-style medicine systems as diuretic, tonic and used to cure or treat fever, indigestion, mild cough and cold, menstrual disorders from ancient period of time.[9] In different geographical regions and languages, there are many vernaculars that *Rheum* has. In common language, *Rheum* is known by the rhubarb, Himalayan rhubarb or Indian rhubarb. In hindi or regional area it is known by Dolu, revandchini, particularly in Garhwal/Kumaon region of Uttarakhand state, it is known by Dolu or Archu.

The taxonomic classification of *Rheum* plant can be explained as:

| | |
|---|---|
| **Kingdom** | **Plantae** |
| **Clade** | **Core eudicots** |
| **Clade** | **Eudicots** |
| **Clade** | **Angiosperm** |
| **Order** | **Caryophyllales** |
| **Family** | **Polygonaceae** |
| **Genus** | ***Rheum*** |
| **Species** | ***emodi*** |

*Rheum* genus consists of about 60 species worldwide and among them around 10 species are belonged to the Indian northern Himalayan region. Some of the major species of *Rheum* with their geographic locations are labelled in the table **(Table. 1.1)** by the Upadhyay and coworkers.[10]

*Rheum emodi* is cultivated and naturally grown in the temperate region of Himalayas, from Kashmir to the eastern states of the India. It is generally grown at a height of 2500-4000 M. Rhubarb found majorly in alpine zones on the crevices, rubbles stream and rocky soil in specific areas. Rhubarb can grow upto a height ranging from 1.5 to 3 M, with a thick stem and broad leaves. Leaves are large radical, broadly ovate, orbicular with a diameter of 30-40 cm with long petioles. The stems are greenish in

colour, with brown streaks. Flowers are small, dark purple to light red in colour. The plant has a reproductive phase after the 3-5 years of juvenile phase.

Flowering occurs in the month of May to July. Fruits are ovoid, winged, of about 1-2 cm long with a arose apex. Seeds are also winged and were collected in the month of August- September after they turned to a dark brown in colour. Potential of seeds production may vary from plant to plant, ranging from 300 seeds to 950 seeds per plant. Rhizomes are light orange to yellowish brown in colour with a length of 5-10 inches **(Fig. 1.1).** Roots are thick and dark brown in colour.[11]

**Table. 1.1. Different *Rheum* species and their geographical distribution.**

| S. No. | *Rheum* spp. | Geographical distribution |
|---|---|---|
| **1** | *R. tanguticum* | China (Quinghai province) |
| **2** | *R. ribes* | Turkey and Iran (Asian countries) |
| **3** | *R. lobatum* | Central Asian countries |
| **4** | *R. macrocarpum* | |
| **5** | *R. turkestanicum* | |
| **6** | *R. spiciforme royle* | Kashmir valley, India |
| **7** | *R. palmatum* | China, Asian countries |
| **8** | *R. alexandrae* | Yunnan, China |
| **9** | *R. emodi* | Indian Himalayan region |
| **10** | *R. austral* | China, India, Nepal, Pakistan |
| **11** | *R. nobile* | Sikkim Himalayas, India |
| **12** | *R. corodatum* | Central Asian countries |
| **13** | *R. wittrockii* | Kazakhstan |
| **14** | *R. maximowiczii* | Uzbekistan |
| **15** | *R. rhaponticum* | Norway, Bulgaria, Europe |

| | | |
|---|---|---|
| **16** | *R. moorcroftianum* | Indian- trans-Himalaya |
| **17** | *R. tataricum* | Asia, Europe |
| **18** | *R. rhabarbarum* | Tibet and China |
| **19** | *R. webbianum* | Jammu and Kashmir, Leh and Zanaskar valley, India |
| **20** | *R. undulatum* | Korea |

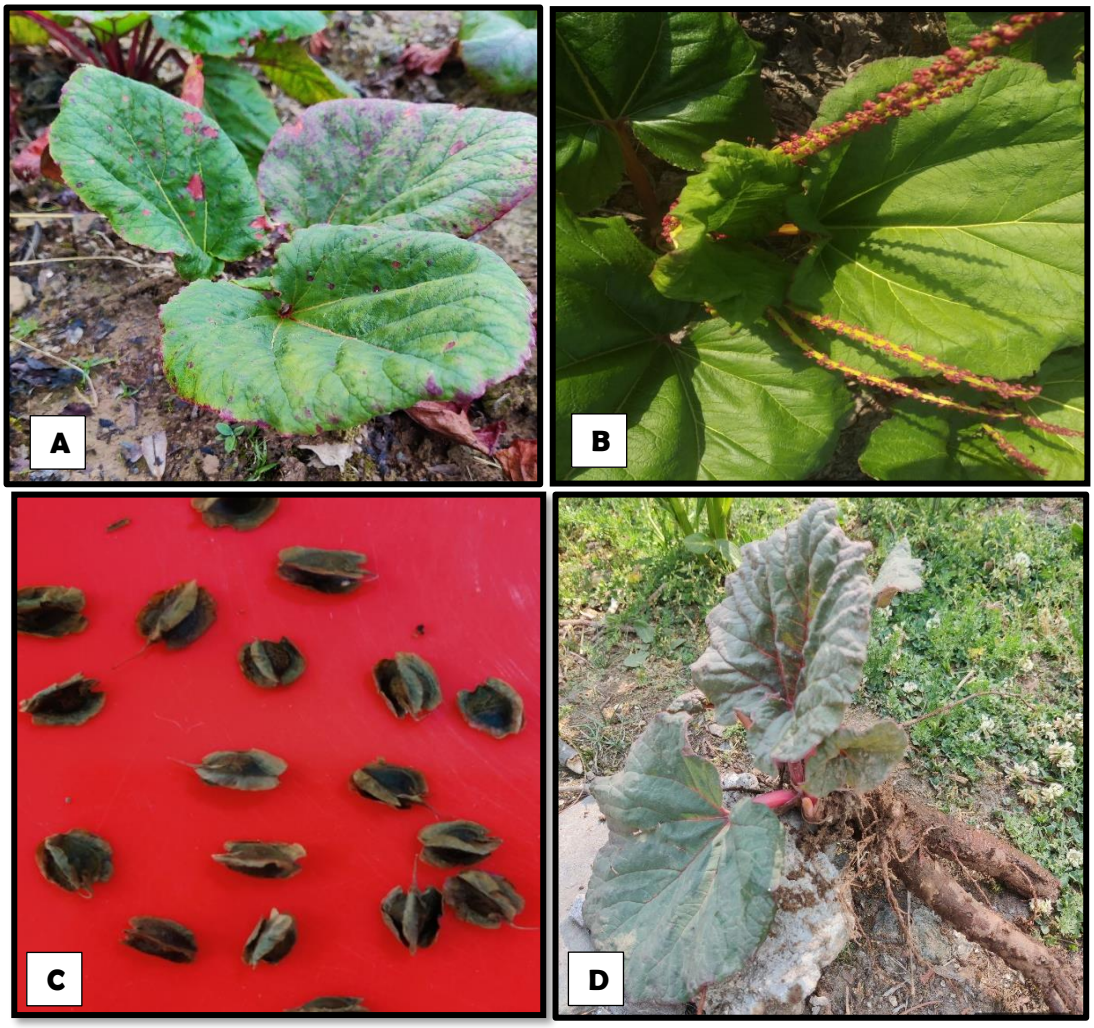


**Fig. 1.1: (A): Juvenile plants of the *Rheum emodi* (B): Flowering plant of *R. emodi* (C): Seeds of *Rheum emodi* (D): Rhizome of *R. emodi***

## 1.3 Therapeutic uses

*Rheum* has a variety of active compounds such as anthraquinone, anthrones, phenols, stilbenes, rhein, emodin, aloe emodin, oxalic acid, flavonoids, chrysophanol etc. Because of all these compounds, *Rheum* considered as a potential disease treating herb.

The anthraquinones derivatives that are present in rhizomes of *Rheum emodi* have various biological activities including antioxidant, nephroprotective activities, anti-bacterial, antifungal, antimicrobial, anti-inflammatory properties. Other compounds are also found such as Oxanthrone derivatives includes revandchinone-4 (the Oxanthrone ether) and revandchinone-1 along with the revandchinone-2 and revandchinone-3 (Oxanthrone esters). Beside these, *Rheum* also contains some complex compounds like sulfemodin 8-O-b-D-glucoside, 6-methyl-rhein, 6- methyl aloe-emodin, Rhein11-O-b-D-glucoside, sulfated anthraquinoneglycoside and Torachrysone 8-O-b- D-glucoside. *Rheum's* rhizomes extract also contained a stilbene compound, resveratrol that has high antioxidant properties which is ranges between 229.4 to 371.7 µg per plant.[12]

The majority of these substances could increase the accessibility of antibacterial and antifungal medications. The methanolic extracts of numerous species of *Rheum* plant were studied and observed by Rolta and coworkers for their phytochemical profiling which includes phenolic content, antioxidant activity, flavonoid content.[13,14] Rhubarb and its parts have been used since ancient old-style system of medicines in different forms; roots in powdered form, rhubarb extraction, crushed leaves and roots for treating and curing several wounds and disease. *Rheum* and its parts are effective in many diseases related to gastro, skin, respiratory system, urogenital system, and muscoskeleton system. Solvent extract of roots of funnel bulbs, celery plant along with the rhubarb extraction can be effective for treating Jaundice. Menorrhagia can be treated when rhubarb was taken continuously for the starting 3 days of menstruation. Hepatic inflammation was treated with the oil of rhubarb; abscess can be cured when rhubarb powder is taken with the liquid extract of wild ginger.[15] The rhubarb leaves could be poisonous or toxic too, as its leaves contains oxalates in high concentration,

as compared to its roots and petioles. A concentration of about 5gm is considered as minimum lethal dose for an adult.[44] As *Rheum* is considered a beneficial plant among the herbal and traditional medicine system, local communities use this plant in daily care routine.

In addition to its medical use, *Rheum* has been used in preparing jelly, jams and sauces as it has slight tangy flavour, along with this its leaves are used for preparing vegetables and salad.[5]

## 1.4 Conservation strategies

In terms of *Rheum* propagation, its seeds have a poor germination percentage, its natural regeneration and growing power is very low. Excessive extraction and low germination and growing potential are the main reasons for the destruction to this herb in its natural habitat. In upcoming years, all the above reasons lead to the decrease in gene flow as well as the gene erosion in *Rheum emodi*. Thus, it become essential to conserve this herbal species.

As a primary conservation method, in situ conservation is used for preserve, protect, maintain and recover the endangered to vulnerable plant species. This conservation is mainly referred to conserve the species in their natural habitat. Some common examples for such conservation are biosphere reserve, national parks, sacred forest and sanctuaries. Another method for conserving plant species is ex-situ conservation, method for preserving the population of plants other than its natural habitat. Examples include botanical gardens, genes, pollen or DNA banks, etc. The ex-situ conservation can act as a holdup method for some specific plant species that might be extinct in the nature especially in the human-dominated environment.[16] Modern science now have advance biotechnology approaches beside these traditional conservation strategies such as the ‘in-situ conservation’ and the ‘ex-situ conservation’ methods. They provide the basic potential for extending these traditional methods to a broader range. The advance techniques are primarily developed for agriculture and horticulture

species, but are now increasingly applied to propagating, evaluating, collecting and preserving the endangered or rare species/ plant germplasm.[17]

As *Rheum* species are now considered in the list of endangered species, thus its conservation becomes necessity. Advancements in the science field, particularly in biotechnology, gave the way for conservation and utilization of natural resources. There are several techniques and methods for the conserving the said plant species such as micropropagation, somatic embryogenesis, organogenesis, cryopreservation, protoplast culture, synthetic seed production etc. All these approaches have made easy to conserve the germplasm of the plant species for a longer period of time.[18]

Many well-established plant tissue culture laboratories have now micropropagated the rare species which faces difficulties in propagation through the conventional or natural horticulture techniques. The techniques of Plant tissue/cell culture mentions the aseptic culturing of plant's cells or tissues, seeds, embryos, organs and the protoplast on a nutrient rich media under the most aseptic and sterilized environment, in controlled humidity, photoperiod, temperature, light and other components of nutrient medium to provide the model and controlled growth environment.[19] As a result, the plants obtained were disease-free with high fertility rate, clones of each other, it allows the large-scale proliferation of true-to-type propagules in a short time frame and controlled environment, no time bound or season dependent, regardless of the climate.[20]

Synthetic seed technology or the Syn seed production has found employment and succeeded to different ornamental and medicinal plants. Syn seeds has huge potential and possibilities in preserving somatic embryos, callus, and many others cells and tissues of valued germplasm. Synthetic seeds by encapsulating the plant's callus or tissues, is proved to be good way to preserve, conserve and exchange of germplasm. Production of synthetic seeds provides the advantages as it is not depend upon season for the propagation of elite germplasm, ornamental or the seedless plants with unique trait.[21] The value of synthetic seed expertise is enormous in the mass proliferation of valuable germplasm, in which the vegetative propagation of seeds that is the natural seed propagation is hinder or afflict with incompatibilities. Encapsulation of seeds

provides the benefits in the terms of fast and easy transportation of the plantlets. Syn seed technique is mostly used to produce high-quality micro-propagules with a limiting factor and also, they are cost effective.

Numerous factor which contributes in accomplishment of germination and the development of syn-seeds were the stored time, quality embryos that is matured embryos, and conditions such as temperature, calcium chloride content in relation to the ratio of sodium alginate and the plant growth hormones' concentration. The synthetic or artificial seeds are equal to the traditional natural seeds since the somatic embryos are covered in one or more artificial chemical coatings that resemble the capsule or beads.[22] As in recent era, many attempts have been employed to encapsulate the other vegetative fragments other than embryos for example axillary buds or the shoot tips and the nodal segments.[23,24]

In general, using synthetic seed can help preserve key germplasm such as rare or about to extinct plant species and also helps in enabling the exchange of planting material crosswise the world's research centres.[25] A wild edible plant species of the Polygonaceae family member, *Rumex vesicarius L*. is often propagated through the most effective way that is through the seeds. These natural seeds are known as recalcitrant or dormant having a very low germination rate of 20 % and very low survival possibility, the mass induction and generation of embryos is essential in these situations.[26] An encapsulation of the somatic embryos to create the artificial or syn seeds has demonstrated to have numerous applications. This methodology is further noted and gain success in other plants also such as '*Ocimum* species' [27]; '*Adhatoda vesica'* [28]**;** '*Allium sativa'* [29]**;** '*Tylophora indica'* [30]**;** '*Daucus carota'* [31]**;** '*Urginea altissima'*.[32]

During the propagation method or the plant tissue culture, *in vitro* plantlets is often cooperated with the genetic instability due to some fundamental factors that includes the mode of regeneration, plant species or varieties involved, ploidy level and genotype, composition and concentration of the growth medium and growth regulators, culture duration and the callus phase duration. In the tissue culture processes, due to the stress to the explant, it goes through the genomic shock and thus

requires reformation of their genomes. Numerous investigations and scientific advances point to a variety of biochemical, morphological, and genetic techniques that might be employed to evaluate the somaclonal variation in various plant species, leading to different effectiveness being seen. In recent years, molecular approaches are being considered as alternatives for the improvement and conservation of various plants through the genetic profiling. Different markers have been established for the authentication of different plants species. The disadvantages associated with the somaclonal variation are that it generates unpredictable and uncontrollable variations that have no use, most of the variation are cultivar independent and the genetic changes that were occur during the regeneration takes place at unstable frequencies and also not heritable, somaclonal variants are not novel and are only possible with plants that have the ability to reproduce themselves repeatedly and grow into whole plants. The somaclones have low fertility rate and reduced growth percentage.

There are many techniques that are known for assessing the somaclonal variation within the regenerated plantlets such as morphological assessment, biochemical assessment, protein markers, detection of somaclonal variation using RFLP, RAPD, ISSR markers etc. A quality check for the genetic or clonal fidelity at very beginning of propagation is helpful and mostly useful in all the plant multiplication techniques. Amongst the numerous suitable DNA-based markers or the molecular markers like RFLP and AFLP are considered as the most commonly used markers for assessing the genetic fidelity in micropropagated floras, as the RAPD and the ISSR markers uses a minor quantity or less quantity of the initial DNA sample; and they don't involve any radioactive markers, and thus are additionally easy to handle, quick, cost-effective and very reliable markers, and other approaches demand great care along with the usage of radioactive labelling of extremely expensive enzymes, making them incompatible under some circumstances. The somaclonal variation in many ornamentals, therapeutic plants have been successfully detected by using these markers.[33,34]

## 1.5 Research Hypothesis

The practice of herbal medicines is growing globally in the modern world. Their devastation of habitat and loss of genetic variety are mostly caused by their increased use and unrestricted harvesting. Therefore, to accommodate the increasing demand for medicinally significant plants, various biotechnological tools are employed, such as tissue culture techniques, synthetic seed development, which aid in the multiplication of medicinal plants and improve their genetic makeup, ultimately producing the high-quality plant-based medicines. Because of its therapeutic value and endangered status, *Rheum emodi* is in high demand, which makes its conservation even more important.

In *Rheum emodi,* the vegetative propagation is less successful. The seeds have an extremely short seedling life; seeds that are a year-old exhibit very poor germination. Excessive extraction and low germination and growing potential are the main reason for the destruction to this herb in its natural habitat. Since seed variability and germination rates are limited. High quality planting materials cannot be obtained only through the seed multiplication for long term use. Mass propagation can be a possible way for fulfilling the demand. True-to-type propagation, also known as *in vitro* propagation uses aseptic culture methods for the selected genotypes. The technology of *in vitro* propagation is crucial in the production of high-quality plant-based medication, virus free products and to conserved the desired genotype. For the conserving aspect of this endangered plant species, production of synthetic seeds can be proved to be an ideal, better and sustainable method.

The present study focused in the mass multiplication of *Rheum emodi* through the leaf explant to fill the gap created by the environmental changes and lack of planting material. For conserving this endangered plant species, production of synthetic seeds can be proved to be an ideal, better and sustainable method. The purpose of this study is to establish the clonal propagation method of herbal plant species of Uttarakhand "*Rheum emodi*" and further assessing its genetic stability throughout the regenerated plantlets.

Focusing on the aforementioned research hypothesis and the research gap, the current study would be focused on the following objectives as:

- *In vitro* regeneration of *Rheum emodi*. Aim is to standardize the sterilization techniques, the media components and plant growth hormones for the optimum growth of plantlets.
- To develop the suitable growth media and hormones concentration for *in-vitro* rooting. Establishing suitable conditions for the acclimatization and hardening of plants raised from tissue culture technique.
- To produce the synthetic seeds and to estimate their viability and germination potential.
- Further to screen the somaclonal variation using the molecular markers such as RAPD (Randomly amplified polymorphic DNA) and ISSR (Inter simple sequence repeats). These DNA based molecular markers are extensively used in assessing the genetic fidelity/clonal fidelity among the regenerants.