India has long and ancient history on the use of medicinal plants in Ayurveda, Unani, and Sidha and still greatly depends on the forest plants. Due to excessive cutting and collection the forest wealth has depleted significantly and over 400 medicinal plant species have acquired the status of vulnerable type (Singh, 2006). *Podophyllum hexandrum* is one of such heavily exploited medicinal “keystone species”. The population size of this medicinal herb is very small (40-700 plants per location) and is declining each year owing to anthropogenic activities and overexploitation (Sharma et al., 2000). Hence, it is essential to have programmes for conservation of endangered and rare medicinal plants.

*Podophyllum hexandrum* Royle is an endangered medicinal plant in the western Himalayas and is heavily exploited for pharmaceutical purposes. The population size of this plant is declining each year. The problem is further aggravated as regeneration of the plant under natural condition is very low. Apparently, there is considerable variation in the morphological characters of this plant such as plant height, leaf characteristics, fruit weight, seed colour etc.

Chatterjee, (1953) described this plant as having 2 or 3 leaves, orbicular-reniform, palmate, pellate with lobed segments, fruit is oblong or elliptical berry with many seeds embedded in the pulp. The species exhibits certain amount of variation in its botanical features. They have been classified into three or four varieties viz. var. *hexandrum*, var. *axillaries*, var. *bhootanensis*, var. *jaeschkei*.

Extracts of *Podophyllum* species have been used for diverse cultures since ancient times as antidotes against poisons, vesicant and suicide agents. Podophyllin was included in the U.S. Pharmacopoeia in 1820, and the use of this resin for the treatment of venereal warts was described, attributing this action to podophyllotoxin. The destructive effect of this resin on experimental cancer cells in animals was also reported (Gordaliza, 2006).

Podophyllotoxin is the most abundant lignan isolated from Podophyllin, the resin obtained from species of the genera *Podophyllum* (Ayers, 1990). Lignans are a family of natural products that originated as secondary metabolites. There are different biological activities in lignans like properties against leishmaniosis, antifungal, antirheumatic, antipsoriasis, antimalarial, antiasthmatic etc. But cytotoxicity and
antiviral are the most important activities that maintain the interest in podophyllotoxin and its analogs.

Combination therapies are currently implemented with other chemotherapeutic agents useful in the fight against cancer and viral infections; multiple myeloma responds best to homeotherapy with podophyllotoxin.

The cytotoxic activity of podophyllotoxin is based on its ability to inhibit the microtubule assembly during cell division. It is used as the starting material for the chemical synthesis of anticancer drugs, etoposide (VP-16-213) and tenoposide (VM-26) (Canel et al., 2000). These drugs act as inhibitors of topoisomerase II and are widely used in the treatment of lung and testicular cancer (Hollithuis et al., 1988). Podophyllotoxin is also produced in relatively minute quantities by other plant species, viz. *P. peltatum*, *P. versipelle*, *Linum flavum*, *L. album* and *Juniperus chinensis* (Jackson et al., 1984; Uden et al., 1989).

The podophyllotoxin and its derivatives etoposide, etopophous (etoposide phosphate), and tenoposide are successfully utilized in the treatment of a variety of malignant conditions. It is also used as a precursor for the chemical synthesis of anticancer drug etoposide, teniposide and etopophous (Farkya et al., 2004). These compounds have been used for the treatment of lung and testicular cancer as well as certain leukemias (Stahelin & Wartburg 1991; Imbert, 1998).

### 2.1 History of plant cell culture technology

At the beginning of the century, Haberlandt (1902) attempted to cultivate isolated plant cells, but cell division was never observed in these cultures. In the 1930s the first *in vitro* cultures were established (White, 1934; Gautheret, 1939), and this was followed by a period of development of culture media and of cultivation methods (Street, 1977). The earliest detailed reference to plant cell cultures as an industrial route to natural product synthesis is probably the patent application of (Routier and Nickell, 1956). Zenk et al., (1985) demonstrated, for the first time, that completely dedifferentiated cell suspensions of a higher plant (*Morinda citrifolia*) can produce secondary metabolites (anthraquinones). However, the low yield of secondary metabolites in suspension cultures clearly was a bottleneck for commercialization. In those early efforts, plant cells in culture were treated in direct analogy to microbial
systems, with little knowledge of plant cell physiology and biochemistry, or the influence of bioreactor operation on the physiological state of such systems. In 1982, at least 30 compounds were known to accumulate in plant culture systems in concentrations equal to or higher than that in the whole plant (Staba, 1982).

2.2 Production of secondary metabolites using plant tissue culture

The large scale plant cell and tissue cultures have been considered as an alternative source of biochemicals over the last 40 years. Routier and Nickel received the first patent for the cultivation of plant tissue in 1956 and suggested its potential for the production of secondary metabolites (Scrugg, 1991). Studies on secondary metabolites plant have been increasing over the last 50 years. These molecules are known to play a major role in the adaptation of plants to their environment, and also represent an important source of active pharmaceuticals. Plant cell culture technologies were introduced at the end of the 1960s as a tool for both studying and producing plant secondary metabolites. Many studies have been undertaken with the objective of improving the in vitro production of plant secondary compounds. Undifferentiated cell cultures such as callus and cell suspension have been mainly studied, but a large interest has also been shown in hairy roots and other organ cultures (Bourgaud et al., 2001). Among the techniques employed, manipulation of nutrient media, optimization of culture conditions, identification of the most effective elicitors and the use of hairy root culture have been given considerable attention.

Unlike humans and animals, plants are not mobile which makes them very susceptible to attack from pests and predators. To overcome this problem, during metabolism plants produce enormous number of compounds as part of defence mechanism (Oksman et al., 2004, Bennett and Wallsgrove, 1994). These compounds are not essential for primary functions like growth, photosynthesis and reproduction and are called secondary metabolites. Secondary metabolites are used as pharmaceutical, agrochemicals, aromatics and food additives (Oksman et al., 2004, Rao et al., 2002). Plant derived compounds include many terpenes, polyphenols, cardenolides, steroids, alkaloids and glycosides (Stafford et al., 1986, Matkowski, 2000). Figure 2.1 shows different groups of chemical compounds produced by plants. The chemical synthesis of many of these metabolites is only possible in plants (Rao et al., 2002), resulting in over exploitation and threat of extinction to many medicinal plant species (Oksman et
Tissue culture offers an effective and potential alternative of metabolite production because the amount of secondary metabolites produced in tissue cultures can be even higher than in parent plants (Rao et al., 2002, Parr, 1989). Advances in plant tissue culture have enabled commercial scale production of plant metabolites.

**Phenylpropanoids**

1. Anthocyanins
2. Coumarins
3. Flavonoids
4. Isoflavonoids
5. Hydroxycinnamoyl Derivatives
6. Lignans
7. Phenolenons
8. Proanthocyanidins
9. Stilbenes
10. Tanins

**Alkaloids**

1. Acridines
2. Quinolizidines
3. Betalaines
4. Furanoquinones
5. Harringtonines
6. Isoquinolines
7. Indoles
8. Purines
9. Pyridines
10. Tropane alkaloids

**Medicinal Plants**

- **Quinones**
  1. Anthroquinones
  2. Benzoquinones
  3. Naphthoquinon

- **Terpenoids**
  1. Carotenes
  2. Monoterpenes
  3. Sesquiterpenes
  4. Diterpenes
  5. Triterpenes

- **Steroids**
  1. Cardiac glycosides
  2. Pregnenolone
  3. Derivative

Since total chemical synthesis of podophyllotoxin is complicated and expensive biotechnological approaches particularly plant cell and tissue cultures appear to be attractive alternatives for the production of this pharmaceutically important lignan. Induction of callus culture from *P. peltatum* and detection of podophyllotoxin from such cultures was first reported by Kadkade (1981, 1982). Uden et al., (1989) initiated podophyllotoxin producing callus cultures from *in vitro* plantlets of the Indian *Podophyllum*; dark-grown cultures accumulated up to 0.3% podophyllotoxin (dry weight basis). After several tedious trials they initiated and established callus cultures of the Indian *Podophyllum* from *in vitro* grown auxenic seedling explants and roots and rhizomes isolated from 1 year old mature plants on B5 and MS media.
supplemented with growth regulators (Majumdhar and Jha, 2007). Podophyllotoxin could be detected from all the callus lines that survived after 1 year of initiation, induced from different juvenile and mature explants (Majumdar and Jha, 2007). Callus cultures producing podophyllotoxin have also been initiated from needles of Callitris drummondii, (Uden et al., 1990) and leaves of Juniperus chinensis (Muranka et al., 1998). Suspension cultures have been proposed to be a viable alternative for the production of economically important phytochemicals. Such cultures have a relatively fast growth rate and are easy to manipulate. Suspension cultures producing podophyllotoxin were initiated from different plant species which are tabulated below (Table 2.1).

Table 2.1 Podophyllotoxin production in cell suspension culture of different plant species

<table>
<thead>
<tr>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Podophyllum hexandrum</em></td>
<td>Majumdhar (2008), Chattopadhyay et al., (2001)</td>
</tr>
<tr>
<td><em>Callitris drummondii</em></td>
<td>Uden et al., (1990)</td>
</tr>
<tr>
<td><em>Linum album</em></td>
<td>Smollny et al., (1998)</td>
</tr>
<tr>
<td><em>L. nodiflorum</em></td>
<td>Konuklugil et al., (1999)</td>
</tr>
<tr>
<td><em>L. mucronatum</em> spp armenum</td>
<td>Konuklugil et al., (2001)</td>
</tr>
</tbody>
</table>

For plant cell culture techniques to become economically viable, it is important to develop methods that would allow for consistent generation of high yields of products from cultured cells (Berlin and Sasse, 1985). The production of some important plant pharmaceuticals produced in cell cultures are summarised in Table 2.2.
### Table 2.2 Bioactive secondary metabolites from plant cell culture

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Active ingredients</th>
<th>Culture type</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agave amaniensis</em></td>
<td>Saponins</td>
<td>Callus</td>
<td>Andrijany et al., 1999</td>
</tr>
<tr>
<td><em>Ailanthus altissima</em></td>
<td>Alkaloids</td>
<td>Suspension</td>
<td>Anderson et al., 1987</td>
</tr>
<tr>
<td><em>Anchusa officinalis</em></td>
<td>Rosmarinic acid</td>
<td>Suspension</td>
<td>De-Eknamkul and Ellis, 1985.</td>
</tr>
<tr>
<td><em>Aloe saponaria</em></td>
<td>Tetrahydroanthracene glucosides</td>
<td>Suspension</td>
<td>Yagi et al., 1983.</td>
</tr>
<tr>
<td><em>Brueca javanica</em> (L.) Merr.</td>
<td>Canthinone alkaloids</td>
<td>Suspension</td>
<td>Liu et al., 1990.</td>
</tr>
<tr>
<td><em>Bupleurum falcatum</em></td>
<td>Saikosaponins</td>
<td>Callus</td>
<td>Wang and Huang, 1982</td>
</tr>
<tr>
<td><em>Capsicum annuum</em> L.</td>
<td>Capsaicin</td>
<td>Suspension</td>
<td>Johnson et al., 1990</td>
</tr>
<tr>
<td><em>Cassia acutifolia</em></td>
<td>Anthraquinones</td>
<td>Suspension</td>
<td>Nazif et al., 2000</td>
</tr>
<tr>
<td><em>Catharanthus roseus</em></td>
<td>Indole alkaloids</td>
<td>Suspension</td>
<td>Moreno et al., 1993</td>
</tr>
<tr>
<td><em>Catharanthus roseus</em></td>
<td>Catharanthine</td>
<td>Suspension</td>
<td>Zhao et al., 2001</td>
</tr>
<tr>
<td><em>Citrus sp.</em></td>
<td>Naringin, Limonin</td>
<td>Callus</td>
<td>Barthe et al., 1987</td>
</tr>
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<td><em>Coffea arabica</em> L.</td>
<td>Caffeine</td>
<td>Callus</td>
<td>Waller et al., 1983</td>
</tr>
<tr>
<td><em>Digitalis purpurea</em> L.</td>
<td>Cardenolides</td>
<td>Suspension</td>
<td>Hagimori et al., 1982</td>
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<tr>
<td><em>Dioscorea deltoidea</em></td>
<td>Diosgenin</td>
<td>Suspension</td>
<td>Heble and Staba, 1980</td>
</tr>
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<td><em>Dioscorea doryophora</em> Hance</td>
<td>Diosgenin</td>
<td>Suspension</td>
<td>Huang et al., 1993</td>
</tr>
<tr>
<td>Plant Species</td>
<td>Secondary Metabolites</td>
<td>Tissue Type</td>
<td>Source</td>
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<td>-------------------------------------</td>
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<td><em>Eriobotrya japonica</em></td>
<td>Triterpenes</td>
<td>Callus</td>
<td>Taniguchi et al., 2002.</td>
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<tr>
<td><em>Eucalyptus tereticornis</em> SM.</td>
<td>Sterols and Phenolic compounds</td>
<td>Callus</td>
<td>Venkateswara et al., 1986.</td>
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<td><em>Eucommia ulmoides</em></td>
<td>Chlorogenic acid</td>
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<td>Wang et al., 2003.</td>
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<td>Secoiridoid glucosides</td>
<td>Callus</td>
<td>Skrzypczak et al., 1993.</td>
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<td>Carrier et al., 1991.</td>
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<td>Kitamura et al., 1998.</td>
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<td>Ayabe et al., 1986.</td>
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<td>Yamada and Hashimoto, 1982.</td>
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<td><em>Linum flavum</em> L.</td>
<td>5-Methoxypodophyllotoxin</td>
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<td><em>Lithospermum erythrorhizon</em></td>
<td>Shikonin derivatives</td>
<td>Suspension</td>
<td>Fujita et al., 1981.</td>
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<tr>
<td><em>Lithospermum erythrorhizon</em></td>
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<td>Fukui et al., 1990.</td>
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<tr>
<td>Plant Species</td>
<td>Compound(s)</td>
<td>Culture Type</td>
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<td><em>Lycium chinense</em></td>
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<td>Suspension</td>
<td>Wichers et al., 1993.</td>
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<td>Callus</td>
<td>Tabata and Hiraoka, 1976.</td>
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<td>Callus</td>
<td>Thengane et al., 2003.</td>
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<td>Furuya et al., 1973.</td>
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<td>Thebaine</td>
<td>Callus</td>
<td>Day et al., 1986.</td>
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<td><em>Papaver somniferum L.</em></td>
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<td>Callus</td>
<td>Furuya et al., 1972.</td>
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<td>Suspension</td>
<td>Siah and Doran, 1991.</td>
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<td>Sakuta et al., 1987.</td>
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<td>Callus</td>
<td>Stojakowska and Kisiel, 1999</td>
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<td><strong>Solanum chrysotrichum</strong> (Schldl.)</td>
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<td>Suspension</td>
<td>Villarreal et al., 1997.</td>
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<td>Suspension</td>
<td>Chandler and Dodds, 1983.</td>
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<td><em>Solanum paludosum</em></td>
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<td>Badaoui et al., 1996.</td>
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<td><em>Stizolobium hassjoo</em></td>
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<td>Huang et al., 2002.</td>
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<td><em>Tabernaemontana divaricata</em></td>
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<td>Sierra et al., 1992.</td>
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<td>Wu et al., 2001.</td>
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<td>Cusido et al., 1999.</td>
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<td><em>Taxus cuspidate</em></td>
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<td>Suspension</td>
<td>Ketchum et al., 2003.</td>
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<tr>
<td><em>Tecoma sambucifolium</em></td>
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<td>Callus</td>
<td>Pletsch et al., 1993</td>
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<td>Berberin</td>
<td>Suspension</td>
<td>Kobayashi et al., 1987.</td>
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<tr>
<td><em>Thalictrum minus</em></td>
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<td>Suspension</td>
<td>Nakagawa et al., 1986.</td>
</tr>
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<td><em>Torreya nucifera var. radicans</em></td>
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<td>Suspension</td>
<td>Orihara et al., 2002.</td>
</tr>
<tr>
<td><em>Trigonella foenumgraecum</em></td>
<td>Saponins</td>
<td>Suspension</td>
<td>Brain and Williams, 1983.</td>
</tr>
</tbody>
</table>

Plant cell culture is a flexible system that is easily manipulated to increase product yields (Roberts and Shuler, 1997). Cultures of plant cells are not limited by
environmental, ecological or climatic conditions (Zhong et al., 1995). The capability
to cultivate plant callus cells and organs in liquid media has also made an important
contribution to modern plant biotechnology with respect to the production of
commercially valuable compounds (Su and Lee, 2007). Cell suspension culture has
more immediate potential for industrial application than plant tissue or organ cultures,
due to the extensive body of expertise acquired from submerged microbial culture
studies (Kieran et al., 1997). Plant cell culture as a production strategy is gaining even
more importance when combined with the consumer demand towards natural products
and additives (Taticek et al., 1991). Moreover, increased pharmaceutical applications
such as phytotherapy can boost the industrial application of plant cell culture. Since
genetic manipulation of cell cultures has a great potential for altering the metabolic
profile of plants, profitability on industrial scale is expected to increase in the future
(Razdan, 2003).

2.3 Research on plant cell culture in India

Inspired by the success of plant cell culture processes in Japan, Germany, and U.S.A.,
a number of Indian companies have now seriously embarked on the research on
production of high value phytochemicals from plant cell and hairy root cultures. India
has a vast flora of medicinal and aromatic plants and rich traditions of using herbal
medicines for treatment of various ailments. However, the scarce availability of plant
material made the Ayurvedic practices difficult. Realizing the untapped resource of
the germplasm, several industrial, R&D and academic institutions in India launched a
number of projects on plant cell cultures. Dabur India Ltd., New Delhi projected the
commencement of a plant for the production of taxol, an anti-cancer drug, from the
cell cultures of Taxus spp. by the year 2000. Cipla Pharmaceuticals Pvt. Ltd.,
Bangalore is striving hard to produce diosgenin and other steroids using the plant cell
culture technology. Dr. Reddy’s Research Foundation, Hyderabad is similarly
interested in taxol and other medicinal compounds such as camptothecin. Central
Institute of Medicinal and Aromatic Plants, Lucknow has reported the successful
culturing of hairy roots for obtaining commercially important medicinal compounds
(Kukreja, 1996). The group at Regional Research Laboratory, Trivandrum has been
working on development of Catharanthus roseus to obtain anti-cancer drugs. Table
2.3 lists various institutions in India producing pharmaceutical compounds. While the
private sector mainly concentrated on medicinal compounds, Central Food Technological Research Institute, Mysore pioneered the research on production of food value metabolites from plant cell and hairy root cultures (Ravishankar and Venkataraman, 1990). Saffron ingredients from *Crocus sativus* cell cultures, anthocyanin from *Daucus carota* cell cultures, betalaines from hairy root cultures of *Beta vulgaris* and capsaicin from immobilized *Capsicum* spp cells are the few processes at different levels of scale-up.

**Table 2.3 Research on plant cell culture in India for the Production of secondary metabolites**

<table>
<thead>
<tr>
<th>Company/University/Institution</th>
<th>Products</th>
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<td>Anthocyanin, capsaicin, betalaines, saffron metabolites, vanilla flavour</td>
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Some efforts have been made to culture cells *in vitro* and produce podophyllotoxin *in vitro* so that precious plant species can be conserved more efficiently and pressure on natural plant can be reduced.

Kadkade, (1982) reported growth and podophyllotoxin production in callus tissues of *Podophyllum peltatum*. IAA and/or NAA in the presence of kinetin and casamino acids promoted callus growth, while 2,4-dichlorophenoxyacetic acid (2,4-D) yielded
maximum podophyllotoxin. With respect to kinetin, the optimum concentration for callus growth and podophyllotoxin formation was 0.2 ppm, with higher concentrations inhibiting podophyllotoxin biosynthesis. The accumulation of podophyllotoxin was primarily restricted to the rapid growth phase of callus tissues and was strongly affected by the light quality and intensity. The results also indicated that the contents of podophyllotoxin and other lignanes were dependent on the conditions used for callus initiation as well as on the plant parts from which they were derived.

Uden et al., (1989) have reported higher amount of podophyllotoxin production in cell cultures of P. hexandrum kept in dark.

Heyenga et al., (1990) reported production of tumour-inhibitory lignans in callus cultures of Podophyllum hexandrum. Callus cultures have been established from root explants of aseptically-grown Podophyllum hexandrum seedlings. A fully defined medium based on Gamborg's B5 salts supplemented with 2,4-dichlorophenoxyacetic acid, gibberellic acid and 6-benzylaminopurine was effective for both initiation and sustained growth of callus tissue. Cultures produced anticancer lignans podophyllotoxin, 4’-demethylpodophyllotoxin and podophyllotoxin 4-O-glucoside at levels similar to those found in the explant material as assayed by high performance liquid chromatography. The relative proportions of podophyllotoxin and 4’-demethylpodophyllotoxin were markedly influenced by the presence of plant growth regulators. Particularly high levels of podophyllotoxin were associated with growth regulator induced tissue differentiation.

Uden et al., (1990) while studying 5-methoxypodophyllotoxin production in cell suspension of Linum flavum concluded that in lignan-accumulating cultures of L. flavum, PAL activity is nearly always detectable and seems to respond to reciprocal relationship with 5-MPT accumulation.

Uden et al., (1990) while evaluating seven precursors from the phenylpropanoid routing and one related compound in cell suspension cultures derived from rhizome of P. hexandrum obtained 12.8 fold increase in podophyllotoxin production in case of coniferin alone but not when used in combination with isopropanol.
Woerdenbag et al., (1990) reported increased podophyllotoxin production in *Podophyllum hexandrum* cell suspension cultures after feeding coniferyl alcohol as a β-cyclodextrin complex. β-Cyclodextrin itself was proven to be non-toxic for the cells. It did not influence the podophyllotoxin content and it was not metabolized or used as a carbon source by the cells. For comparison, coniferin, the water-soluble β-D-glucoside of coniferyl alcohol, was also fed in the same concentration. The effect of coniferin on the podophyllotoxin accumulation was stronger than that of coniferyl alcohol complexed with β-cyclodextrin, but coniferin is not commercially available.

Uden et al., (1995) reported the production of podophyllotoxin and its 5-methoxy derivative through bioconversion of cyclodextrin-complexed desoxypodophyllotoxin by plant cell cultures in *Linum flavum* and of *Podophyllum hexandrum*. The apolar substrate could be easily dissolved in the culture medium at a concentration of 2 mM by complexation with dimethyl-β-cyclodextrin. Growth parameters of the cell suspensions were not affected by either the addition of cyclodextrin itself, or when cyclodextrin-complexed desoxypodophyllotoxin was present in the medium. The complexed lignan disappeared from the medium within 7 days for both cell cultures. Cellurally only small amounts of desoxypodophyllotoxin were found. After feeding of desoxypodophyllotoxin, the cell culture of *L. flavum* accumulated 5-methoxypodophyllotoxin and 5-methoxypodophyllotoxin-β-D-glucoside. After 7 days a total maximal content of 2.38% on a dry weight basis of 5-methoxypodophyllotoxin was formed corresponding with 249 mg l-1 suspension. The highest bioconversion percentage of 52.3% was found at day 14. The desoxypodophyllotoxin-fed culture of *P. hexandrum* accumulated podophyllotoxin and its β-D-glucoside with a maximal content of 2.87% on a dry weight basis after 9 days, corresponding with 192 mg l-1 suspension. The highest bioconversion percentage of 33.2% was also found at day 9.

Smolly et al., (1998) reported accumulation of podophyllotoxin and related lignans in cell suspension cultures of *Linum album*. Podophyllotoxin and 5-methoxypodophyllotoxin were the main products and were present as glycosides, together with small amounts of deoxypodophyllotoxin, 5′-demethoxy-5-methoxypodophyllotoxin, lariciresinol, pinoresinol, matairesinol, α- and β-peltatin, as well as the monolignol glucoside, coniferin. In dark and light grown cultures,
maximal product yields of lignans of up to 0.2% and 0.5% of the dry weight, respectively, were achieved, mainly consisting of podophyllotoxin.

Muranaka et al., (1998) reported production of podophyllotoxin in *Juniperus chinensis* Callus cultures treated with oligosaccharides and a biogenetic precursor. Calli were induced from the leaves of young trees of *Juniperus chinensis* on Schenk and Hildebrandt medium supplemented with naphthalene acetic acid and kinetin and sub cultured on the same medium. Podophyllotoxin, was isolated from the extractives of calli. The podophyllotoxin in the calli derived from the leaves constituted 0.005% and in intact plant it was 0.0025% of dry weight. The cultures of the calli produced twice as much podophyllotoxin as did those of the intact plant. The production of podophyllotoxin was increased fifteen-fold by addition of chito-oligosaccharides, an elicitor, to the calli. And furthermore, the production increased eleven-fold by addition of phenylalanine, a biogenetic precursor of podophyllotoxin.

Podophyllotoxin production has also been investigated in the rhizome of *P. hexandrum*. (Giri et al., 2000).

Propagation and conservation of *Podophyllum hexandrum* has been reviewed by Nadeem et al., (2000). This report deals with the successful propagation of this species using both conventional and *in vitro* techniques. To improve vegetative multiplication, rhizome segments were treated with α-naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA) before planting; more than doubling in rooting percentage was observed with 100.0 M IBA. Seed germination generally started 3 months after sowing, and pre-treatment with sodium hypochlorite resulted in five-fold improvement, while 250.0 M gibberellic acid (GA₃) and a combination of GA₃ and 6-benzyladenine (BA; 250.0 M each) enhanced germination by nearly two-fold; other treatments were either ineffective or enhanced germination only marginally. Excised zygotic embryos germinated within 7–8 days of culture on basal medium or on medium supplemented with indole-3-acetic acid (IAA; 1.0–4.0 M) and BA (1.0 M). Multiple shoots were formed within 4–5 weeks; following rooting of these shoots, plants were transferred to hardening pots. Somatic embryos were formed from callus derived from zygotic embryos after 4 months of culture on medium containing 5.0 M NAA and 0.5 M BA. Various strategies have been discussed to encourage
cultivation of this medicinal herb so as to reduce pressure on its population in the wild.

Chattopadhyay et al., (2001) studied development of suspension culture of \textit{Podophyllum hexandrum} for production of podophyllotoxin. As the cultures grew, reduction in cell viability, biomass and product yield were associated with browning of culture medium, clumping of cells and drop in medium pH. Supplementation of the medium with both polyvinylpyrrolidone (PVP) and pectinase eliminated these problems. PVP at 10 g l\(^{-1}\) was optimum for both growth of and product formation in \textit{P. hexandrum} suspension cultures.

Canel, (2001) reported high yield of podophyllotoxin from leaves of \textit{Podophyllum peltatum} by \textit{in situ} conversion of podophyllotoxin 4-0-D-Glucopyranoside. Rehydration of powdered tissues of \textit{Podophyllum peltatum} prior to extraction with an organic solvent allows endogenous 0-glucosidases to hydrolyze lignan 4-0-13-D-gluco-sides \textit{in situ} and increase the yield of podophyllotoxin. Aqueous extraction of rhizomes and leaves of \textit{P. peltatum} yielded 4- to 10-fold greater quantities of podophyllotoxin than the traditional ethanolic extraction. Most significantly, leaves were shown to contain over 52 mg of podophyllotoxin per g of dry weight (5.2%), exceeding levels previously reported from any source. These results point to the use of leaves harvested from cultivated \textit{P. peltatum} as an attractive alternative to the destructive collection of natural populations.

Peterson and Alfermann, (2001) studied production of cytotoxic lignans by plant cell cultures. Cytotoxic lignans derived from podophyllotoxin are currently used in cancer chemotherapy. Podophyllotoxin for semi-synthetic derivatization is isolated from the rhizomes of \textit{Podophyllum} plants growing wild, some of which are counted as endangered species. An alternative source for podophyllotoxin or related lignans may in future be cell cultures derived from different plant species, such as \textit{Podophyllum} spp or \textit{Linum} spp. These cell cultures were shown to accumulate considerable amounts of podophyllotoxin or 5-methoxypodophyllotoxin. Optimization of the cell cultivation regime might lead to a renewable source of cytotoxic lignans for medicinal uses. They also attempted to establish plant cell cultures for the production of podophyllotoxin and related lignans and their optimization towards high levels of these target compounds.
Optimization of culture parameters for production of podophyllotoxin in suspension culture was illustrated by Chattopadhyay et al., (2002). Root explants of germinated seedlings were grown on MS medium supplemented with IAA and activated charcoal for obtaining healthy callus after 3 weeks of incubation at 20°C. Problem in cultivation for plant cells in shake flask such as clumping of cells and browning of media was solved by addition of pectinase and polyvinylpyrrolidone. Effect of major media components carbon source was studied on the growth and podophyllotoxin production. It was found that glucose was better carbon source then sucrose. Other culture parameters (inoculums level, pH, IAA, glucose, NH$_4^+$: NO$_3^-$ ratio, and PO$_4^{3-}$) on the overall growth and the product response of the plant cell suspension was further investigated by Plackett-Burman design. This indicated that inoculum level, glucose, IAA, and pH had significant effects on growth and production of podophyllotoxin.

Submerged cultivation of *Podophyllum hexandrum* for the production of podophyllotoxin was carried out in 3 litre stirred tank bioreactor fitted with a low shear steric impeller (Chattopadhyay et al., 2002). The specific requirement of medium, such as carbon source (sugar) and light were established for the growth of and podophyllotoxin production by *Podophyllum hexandrum* in suspension cultures. Substitution of sucrose by glucose resulted in higher growth and podophyllotoxin production. The biosynthesis of podophyllotoxin was favoured when plant cells were cultivated in dark with agitation speed of 100 rpm which was sufficient to mix the culture broth in the bioreactor without causing any significant cell damage.

Premjet et al., (2002) studied the enhancement of podophyllotoxin by biogenetic precursor and elicitors in cell suspension cultures of *Juniperus chinensis*. Suspension cultures were conducted in Schenk and Hildebrandt medium. On addition of phenylalanine and coniferyl alcohol to the cultures, podophyllotoxin was enhanced 2.6 and 6-folds compared with the control respectively. Chito-oligosaccharides, chitopentaose and methyl jasmonate stimulated cell to produce podophyllotoxin at 5, 5.2 and 1.5 times the control level, respectively. The combination of two elicitors, chitopentaose and methyl jasmonate, markedly increased 15-folds relative to the control.
The effect of major medium ingredients (sugar, nitrogen source and phosphate) in *Podophyllum hexandrum* suspension cultures was investigated by Chattopadhyay et al., (2003) in order to increase the production of podophyllotoxin. Amongst, all the culture media such as B5, Eriksson, MS, Nitsch, Street and White’s media, MS medium resulted in comparative growth and enhanced podophyllotoxin accumulation. *Podophyllum hexandrum* has been successfully cultivated in a 3 litre stirred tank bioreactor under low shear condition in batch and fed batch modes of operation by Chattopadhyay et al., (2003b). The concentration of podophyllotoxin in batch mode was found to be 21.4g/l and 13.8 mg/l in 24 and 26 days which were used to identify the mathematical model for developing the design of nutrient feeding strategies of fed batch mode. The model identified the nutrient feeding rate 150 ml/d and substrate uptake rate 105g/l from incoming feed for nonlimiting and noninhibitory glucose concentration in cell retention bioreactor. These optimized parameters have been reported to enhance the biomass and podophyllotoxin accumulation to 48 g/l (DCW) and 43.2 mg/l respectively in 60 days, also when bioreactor was optimized in cell retention cultivated mode biomass and intracellular podophyllotoxin accumulation was 53g/l and 48.8 mg/l after 60 days (Chattopadhyay et al., 2003a,b).

Vardapetyan et al., (2003) studied effect of various elicitors on lignan biosynthesis in callus cultures of *Linum austriacum*. The elicitors under study affected various steps in the metabolic pathway of lignan biosynthesis. Elevated enzyme activity accompanied an elicitor-enhanced synthesis of podophyllotoxins and peltatins.

Lin et al., (2003) reported production of podophyllotoxin using cross-species coculture of *Linum flavum* hairy roots and *Podophyllum hexandrum* cell suspensions. The hairy roots and suspensions were cocultured in Linsmaier and Skoog medium in dual shake flasks and dual bioreactors. In separate experiments, coniferin feeding was shown to be an effective strategy for increasing the accumulation of podophyllotoxin in *P. hexandrum* suspensions. Because roots of *L. flavum* are a natural source of coniferin, hairy roots of this species were used in coculture with *P. hexandrum* to provide an *in situ* supply of coniferin. Compared with *P. hexandrum* suspensions cultured alone in shake flasks or bioreactors, podophyllotoxin concentrations in cocultured *P. hexandrum* cells were increased by 240% and 72% in dual shake flask and dual bioreactor systems, respectively. The availability and stability of coniferin in
the medium are the most likely factors limiting podophyllotoxin synthesis in coculture.

Chattopadhyay et al., (2004) studied cytotoxicity of *in vitro* produced podophyllotoxin from *Podophyllum hexandrum* on human cancer cell line. Podophyllotoxin was produced by cell culture of *Podophyllum hexandrum* under *in vitro* culture conditions. A maximum of 4.26 mg/L of podophyllotoxin was produced when *P. hexandrum* was cultivated in 3L stirred tank bioreactor. The compound extracted from the cell culture was applied to the human breast cancer cell line (MCF-7) and 1 nM podophyllotoxin was able to inhibit the growth of the cancer cells by 50%. The most profound inhibitory effect of podophyllotoxin was observed when it was applied in the beginning of cell growth.

Ardakani et al., (2005) studied effect of elicitors on enhancement of podophyllotoxin biosynthesis in suspension cultures of *Linum album*. Attempts were made to manipulate the biosynthetic pathway of PTOX by some biotic (yeast extract) and abiotic (Ag+, Pb2+ and Cd2+) elicitors for 24 or 48 hr in Murashige and Skoog (MS) medium. Silver significantly enhanced PTOX production up to 0.24% (mg/g cell dry wt.) in cultures. This effect could be attributed to the inhibitory role of silver on production of ethylene.

A simple microanalytical technique has been used by Mishra, et al., (2005) for determination of podophyllotoxin in *Podophyllum hexandrum* roots by quantitative RP–HPLC and RP–HPTLC. Podophyllotoxin in methanol extract was calculated from 1 g to 2 mg of plant material, which ranged from 4.25 to 5.22% and 4.15 to 5.12% by HPLC and HPTLC respectively. Beyond this limit, there were difficulties in extraction and consistency of results. In case of podophyllin, consistent results could be obtained up to 10 mg of plant powder. Podophyllotoxin content in resin was estimated as 4.91–5.57% and 4.90–5.58% by HPLC and HPTLC respectively.

Kranz and Petersen, (2005) studied β-Peltatin 6-O-methyltransferase from suspension cultures of *Linum nodiflorum*. S-Adenosyl-l-methionine:β-peltatin 6-O-methyltransferase was isolated and characterized from cell suspension cultures of *Linum nodiflorum*. The suspension cell line of *Linum nodiflorum* was characterized with respect to growth, medium alterations and lignan production as well as activity
of SAM:β-peltatin 6- O-methyltransferase. Highest specific activities of β-peltatin 6- O-methyltransferase were determined on day 7 of the culture period corresponding to the highest levels of 6-methoxypodophyllotoxin on days 7 to 12.

Srivastava et al., (2005) studied plant-based anticancer molecules. A number of natural products, with diverse chemical structures, have been isolated as anticancer agents. Several potential lead molecules such as camptothecin, vincristine, vinblastine, taxol, podophyllotoxin, combretastatins, etc. have been isolated from plants and many of them have been modified to yield better analogues for activity, toxicity or solubility. Several successful molecules like topotecan, irinotecan, taxotere, etoposide, teniposide, etc. also have emerged as drugs upon modification of these natural leads and many more are yet to come. In this review, the authors have focused on four important anticancer leads, that is, camptothecin, taxol, combretastatin A-4 and podophyllotoxin. Their chemistry, structure and activity relationships, biological activities, modes of action, analogue synthesis and future prospects have been discussed.

Sultan et al., (2006) achieved in vitro propagation of Podophyllum hexandrum through callus culture. Callus initiation occurred from root segments of in vitro grown seedlings on Gamborg’s (1968) medium. Shoot proliferation occurred from callus cultures cultured on basal MS medium supplemented with BAP and IAA either alone or in combination. Regenerated shoots were rooted with high efficiency on MS medium containing activated charcoal and NAA. The rooted plantlets were transferred to green poly house in jiffy pots containing sand, soil and vermiculite in 1:1:1 ratio.

Sharma et al., (2006) observed that the seeds of P. hexandrum, H. niger, I. racemosa and B. Persicum were completely dormant at harvest. The efficacy of chilling, acid scarification, KNO₃ and GA₃ treatments for germination improvement was tested. The most effective treatment in different species were- P. hexandrum: H₂SO₄/10⁻³ M GA₃; H. niger 10⁻³ GA₃; I. racemosa: chilling; B. Persicum : chilling; C. carvi: chilling; S. costus: chilling; R. australe: 10⁻³ M GA₃. The presence of chemical inhibitors in dormant seeds, assessed as the degree of inhibition of seed germination of Triticum aestivum and Brassica juncea was indicated in B. persicum and C. carvi.
The seedlings derived from seeds exposed to the various treatments performed well when grown in glass house.

Bedir et al., (2006) found that crushing injury, damaging the leaves in more than 70% of its area, has improved podophyllotoxin content of leaves when dried at 40°C within 24 h of harvest. In contrast, podophyllotoxin content was greatly reduced when the leaves were dried at room temperature at 15% relative humidity and 24°C. Podophyllotoxin was stable with no significant changes over time when the leaves were dried, ground, and stored under different conditions for up to 60 days. Based on these findings, mayapple leaves do not require careful handling at harvest. In fact, leaves can be handled in a manner consistent with mechanical injury as long as leaves are dried at 40°C within 24 h. Leaves can then be stored for up to 60 days, and probably much longer, when dry. If leaves cannot be dried in a timely manner, they can be stored at 4°C for up to 4 weeks without significant loss of podophyllotoxin.

Mohagheghzadeh et al., (2006) reported quantification of aryltetralin lignans in Linum album organs and in vitro cultures. Concentrations of podophyllotoxin (PTOX) and related aryltetralin lignans α-peltatin, β-peltatin, 5’-demethoxy-6- methoxypodophyllotoxin and 6-methoxypodophyllotoxin (MPTOX) in L. album fresh plant organs were determined by HPLC. A degree of variation was observed in lignan content in different plant parts and growth stages. It was found that PTOX is predominant in productive organs and leaves. The highest amount of PTOX (0.651 mg/100g dry wt.) and MPTOX (0.670 mg/100g dry wt.) and the total quantified lignans were found in the capsules. In vitro cultures were studied for lignans followed by high productive cell line selection. Calli cultures were more productive for MPTOX than PTOX, while suspension cells accumulated comparable amounts of PTOX and MPTOX. The highest amount of PTOX (0.301% mg/g dry wt.) was found in suspension originated immobilized cultures.

Recently, Ahmad et al., (2007) attempted in vitro culture of Podophyllum hexandrum. Aseptically germinated embryos of Podophyllum hexandrum developed on solid nutrient agar slab and callus were developed on MS medium with pH 5.8 and varying concentration of BAP, NAA, GA3. MS medium supplemented with NAA and BAP were ineffective for both initiation and sustained growth of callus tissue. The
podophyllotoxin content analysed from callus by HPLC was 0.78 and HPTLC was 0.79 %.

Kushwaha et al., (2007) reported that GA$_3$ induced changes in slow growing endangered Himalayan plant *P. hexandrum* and hastening of vegetative growth. The plants exhibit delayed emergence of functional leaves or hypocotyl dormancy. However, on GA$_3$ treatment the functional leaves were found to emerge at a favourable temperature of 25°C in higher percentage of seedlings and in a shorter time. Functional leaves emerged even at 10°C, a temperature when hypocotyl dormancy generally prevails. A considerable increase in the biochemical parameters related to carbon and nitrogen metabolism [starch, sugars and soluble nitrates, amylase and nitrate reductase (NR) activity], respiration and total dehydrogenase activity in all the seedling parts also indicated an enhancement of metabolic processes as influenced by GA$_3$, for further growth and development. Specific leaf area of the green cotyledonary leaves increased at 25°C, probably to meet the carbon and nitrogen requirements for new structure formation. Higher activity of enzymes involved in carbon and nitrogen metabolism, i.e., NR and α-amylase especially at Hbn, the region of leaf meristematic activity, was further indicative of higher metabolism for earlier initiation of rapid vegetative growth. Initiation of reserve accumulation was also observed at 25°C.

Kim et al., (2007) studied high frequency plant regeneration via somatic embryogenesis in *Podophyllum peltatum*. Somatic embryos differentiated directly from cotyledon explants of zygotic embryos on Murashige and Skoog (MS) basal medium supplemented with 21.6 mM α-naphthaleneacetic acid after 8 weeks of culture. An embryogenic callus developed from cotyledon explants of somatic embryos on MS medium + 6.78 mM 2,4-dichlorophenoxy acetic acid under continuous darkness after 6 weeks of culture. When the embryogenic callus was first grown on MS basal medium + abscisic acid (11.35 mM) for three weeks, followed by sub-culturing on to plain MS basal medium, it led to the development of high frequency somatic embryogenesis. Germination of cotyledonary somatic embryos was noticed on MS basal medium with the addition of 2.89 mM gibberellic acid after two weeks. The germinated embryos grow into plantlets with well developed roots. Rooted plantlets were acclimatized in soil.
Federolf et al., (2007) reported aryltetralin-lignan formation in two different cell suspension cultures of *Linum album*: Deoxypodophyllotoxin 6-hydroxylase, a key enzyme for the formation of 6-methoxypodophyllotoxin. Suspension cultures initiated from two different *Linum album* seedlings accumulate either podophyllotoxin (PTOX, 2.6 mg/g DW) or 6-methoxypodophyllotoxin (6MPTOX, 5.4 mg/g DW) as main lignans.

Sagar and Zafar, (2008) studied *in vitro* enhanced production of podophyllotoxin in phytohormonal induced and regenerated roots of *Podophyllum hexandrum*. Sixty-day-old *in vitro*–induced roots and roots regenerated from totipotent calli of *Podophyllum hexandrum* produced an enhanced quantity of podophyllotoxin. Induced root cultures originated from aseptically germinated seedlings, and regenerated roots were established on both Gamborg's B5 and Murashige and Skoog media. The highest values, both in growth/proliferation and in podophyllotoxin production, were obtained using Gamborg's B5 medium.

Kharkwal et al., (2008) developed an efficient method of propagation of *P. hexandrum* through vegetative propagation and genetic diversity through seeds in *Podophyllum hexandrum*. When seeds of the 14 accessions that were tested exhibited no dormancy and hence did not require any pre-treatments. Besides accession, collection of seeds with high moisture content could be one of the reasons for lack of dormancy. They proposed germination of seeds (while they still retained moisture) in sand at 25° C for high and reproducible results within a shorter period of time compared with earlier reports. Hypocotyl dormancy is known to considerably delay plant establishment and hence en masse propagation by preventing the emergence of functional leaves for up to 11-12 months. Manual removal of cotyledonary leaves, being labour and time intensive, is not a feasible method for large-scale seedling establishment. However, in this study, authors showed that GA$_3$ at 200 ppm can alleviate hypocotyl dormancy besides reducing the time taken for true or functional leaf emergence. Treatment of cotyledonary leaves of 1 week-old-seedlings with 200 ppm GA$_3$ resulted in true or functional leaf emergence within 7 days, and the resultant plants were also more vigorous than the ones obtained from manual removal of cotyledonary leaves. The study helped advance the establishment of seedlings by one growing season (almost 1 year).
Khuswana et al., (2008) reported temperature-dependent growth and emergence of functional leaves. As an adaptive mechanism, hypocotyl dormancy delays emergence of functional leaf until favorable season of growth in *P. hexandrum*. However, upon exposure of the freshly germinated seedlings to favorable temperature (25°C), functional leaves emerged within 20 days. Therefore, the authors examined regulation mechanisms of growth and development of this alpine plant by temperature under laboratory conditions. The seedlings were exposed to (1) 25°C (temperature prevailing at the time of maximum vegetative growth), (2) 4°C (mean temperature at the onset of winter in its natural habitat), and (3) 10°C (an intermediate temperature). Slackened growth at 4°C was followed by senescence of aerial parts and quiescence of roots and predetermined leaf primordia. Rapid development of leaf primordia at 25°C was associated with increased starch hydrolysis. This was evident from higher alpha-amylase activity and reducing sugars. These parameters decreased on sudden exposure to 4°C. In contrast, the roots (perennating organs) showed a slight increase (1.36-fold) in alpha-amylase activity. Growth and development in seedlings growing at 10°C (temperature less adverse than 4°C) were comparatively faster. The content of reducing sugars and alpha-amylase activity were also higher in all the seedling parts at 10°C as compared to 4°C. Irrespective of temperature, maximum changes in nitrate and nitrate reductase occurred during the initial 10 days, i.e., when the readily available form of sugars (reducing sugar) was highest. This indicated that a temperature-dependent availability of carbon, but not temperature itself, was an important regulator of uptake and reduction of nitrogen.

Baldi et al., (2008) studied improved podophyllotoxin production by transformed cultures of *Linum album*. Various cell and hairy root cultures of *L. album* were developed and analyzed for podophyllotoxin content. The effect of different cultivation media and carbon source on growth and podophyllotoxin production were studied in shake-flask suspension cultures. Detailed batch growth and production kinetics with sugar consumption profile were also established. Maximum volumetric productivity of 4.40 and 2.75 mg/L per day was obtained in cell suspension and hairy root cultures, respectively.

Lin et al., (2008) reported simultaneous determination of podophyllotoxin, quercetin and kaempferol in podophyllin by liquid chromatography tandem mass spectrometry.
Chromatographic separation was performed on a Cosmosil 5C18-MS (25 cm × 4.6 mm I.D., 5 μm) reverse phase column using a gradient of mobile phase (0.25% formic acid-methanol). The column effluent was split 2:3 into the photodiode detector and tandem mass spectrometer. Podophyllotoxin, quercetin and kaempferol in podophyllin were identified by daughter ion scan mode and then determined their contents by multiple-reaction monitoring (MRM) mode. The limits of detection and quantitation for podophyllotoxin, quercetin and kaempferol were 2.40, 8.01; 2.94, 9.87 and 3.10, 10.2 ng/mL, respectively. The relative standard deviations of intraday and interday analyses for podophyllotoxin, quercetin and kaempferol ranged from 0.52-6.01% and 2.48-9.88%, respectively. The mean recoveries for podophyllotoxin, quercetin and kaempferol were 101.4%, 98.3% and 98.7%, respectively. The developed LC/MS/MS method was suitable for the simultaneous determinations of podophyllotoxin, quercetin and kaempferol in podophyllin.

Anbazhagan et al., (2008) reported podophyllotoxin production via cell and adventitious root cultures of *Podophyllum peltatum* they established the embryogenic cell and adventitious root culture systems in *P. peltatum* and analyzed PTOX production. For the growth of embryogenic cell clumps in shake flask culture, the most efficient concentration of 2,4-dichloroacetic acid (2,4-D) was 6.78 μM, and the growth of embryogenic cell clumps was 15.9-fold in Murashige and Skoog MS liquid medium with 6.78 M 2,4-D after 3 week of culture. To induce adventitious roots, half-strength MS medium showed the best results for adventitious root induction compared to full strength MS medium and MS medium lacking NH₄NO₃. Optimal indole-3-butyric acid concentration for adventitious root formation was 14.78 μM. In liquid medium, the frequency of adventitious root formation from root segments was 87.7% and the number of laterally formed adventitious roots was 22.3 per segment. PTOX production in embryogenic cells and adventitious roots was confirmed by liquid chromatography and electro-spray ionization–tandem mass spectrometry analysis. High-performance liquid chromatography analysis revealed that adventitious roots contained higher PTOX than embryogenic cell clumps. Elicitor treatment (20 M methyl jasmonate) strongly enhanced the production of PTOX in both embryogenic cell clumps and adventitious roots. This observation suggests that both embryogenic cell and adventitious root culture can be adopted to produce PTOX.
Li et al., (2009) established a way of producing podophyllotoxin by root culture of *Podophyllum hexandrum* on the basis of aseptic plantlet induction from mature embryos and rhizomes which were excised to 2 cm and then cultured on the MS medium supplemented with varying concentration of growth regulators (IBA, GA₃, Hydroquinone and Activated charcoal). Hydroquinone promoted the plantlet growth as compared to IBA and GA₃ with increase in length of rhizome after 40 days of incubation. MS medium supplemented with activated charcoal could significantly hasten the plant growth for optimal biomass and accumulation of podophyllotoxin in root culture of *Podophyllum hexandrum*.

Li et al., (2009) studied dormancy mechanism and bioactivity of hydroquinone extracted from *Podophyllum hexandrum* seed. Ethyl ether extraction from seed of *P. hexandrum* Royle was separated by GC-MS and nineteen compounds were identified including fatty acid, alkenes, etc. most of which had been proved to have inhibited bioactivities that could be the primary reason of unhealthy germination for *P.hexandrum* seeds. Hydroquinone was one of the nineteen compounds and the first time identified from *P.hexandrum* seed in this experiment which had never been reported about its bioactivity before. The further study of its bioactivities was conducted on wheat and *in vitro* culture of embryo of *P.hexandrum* seed showed that hydroquinone played a positive role and shortened the cycle in growth and development compared to IAA, NAA, BAP, GA₃, which indicated that hydroquinone has similarity to auxin on plant growth and development.

Zheljazkov et al., (2009) studied lignan and nutrient concentrations in American Mayapple. Analyses of American Mayapple leaves collected from 37 Mayapple colonies across 18 states indicated a significant variation in podophyllotoxin, α-peltatin, and β-peltatin content and the presence of chemotypes. Overall, the concentrations of podophyllotoxin, α-peltatin, and β- peltatin in the collected accessions ranged from below detectable levels to 45.1, 47.3, and 7.0 mg/g⁻¹ dry weight, respectively. They classified American Mayapple accessions into seven groups on the basis of concentration of podophyllotoxin. American Mayapple was found to grow on various soil types with a range of pH (4.6 to 7.6) and dissimilar concentrations of phytoavailable soil nutrients. Tissue zinc concentration was positively correlated to podophyllotoxin, whereas soil and tissue phosphorus was
positively correlated to the concentration of α-peltatin. The results from this study may contribute toward the development of high podophyllotoxin-containing varieties of American Mayapple and the development of a new cash crop for American farmers.

Sultan et al., (2009) studied *in vitro* embryogenesis and marker guided analysis of *P. hexandrum*. For *in vitro* embryogenesis, excised embryos germinate within a week of inoculation on Murashige and Skoog basal medium supplemented with different concentrations of plant growth regulators, BA (1.0 - 4.0 mg/l) and IAA (0.5 - 2.0 mg/l). Basal medium with BA concentration (0.5 mg/l) and IAA (1.0 mg/l) showed better results than other combinations and was therefore adopted for further studies. The combination of MS + BA (0.5 mg/l + IAA 1.0 mg/l) supplemented with activated charcoal (0.5 - 1.0%) resulted in optimum growth of *P. hexandrum* plantlets. The study has revealed that maximum amount of the podophyllotoxin (5.97%) and podophyllotoxin b-D glycoside (5.72%) was present in the Podophyllum population collected from Keller (Shopian) and Khilanmarg (Gulmarg) area of Jammu and Kashmir, respectively.

Sultan et al., (2009) studied germplasm conservation and quantitative estimation of podophyllotoxin and related glycosides of *Podophyllum hexandrum*. HPLC profiles revealed that all *P.hexandrum* accessions collected from different geographical locations are chemically highly diverse. Authors have observed that chemotaxonomic studies clearly demonstrated that chemical characters of the *P.hexandrum* are suitable to generate essentially the same relationship as revealed by RAPD analysis.

Verma et al., (2009) studied effect of storage containers on seed germination and viability in *Aconitum heterophyllum* and *P. hexandrum*. The study revealed that maximum germination (92%), viability (95%), germination speed (1.58), germination energy (70.0%) were recorded in polybags under complete darkness in *Aconitum heterophyllum* while minimum were recorded under partial light in cloth bags. Similarly, in case of *P. hexandrum* same results were obtained.

Gawde et al., (2009) reported dual extraction of essential oil and podophyllotoxin from *Juniperus virginiana*. The leaves (needles) of eastern red cedar (*Juniperus virginiana* L.) contain two important natural products: essential oil and
podophyllotoxin. Podophyllotoxin was obtained from the leaves following steam
distillation of the leaves to produce the essential oil, indicating that steam distillation
did not degrade podophyllotoxin. Furthermore, a product with 6% purity
podophyllotoxin was obtained from the steam-distilled plant material, demonstrating
the possibility for the establishment of an industrially economic protocol for dual
extraction of these two natural products. This study demonstrated that *J. virginiana*
leaves, currently a waste-product from the timber industry, could be sequentially
extracted for essential oil and podophyllotoxin and utilized as a by-product instead.

Yousufzadi et al., (2010) reported that salicylic acid improves podophyllotoxin
production in cell cultures of *Linum album* by increasing the expression of genes
related with its biosynthesis. Treatment of *L. album* cell cultures with 10 μM salicylic
acid for 3 days improved podophyllotoxin (PTOX) production up to 333 μg/g dry
weight over three times that of the control cultures.

Sultan et al., (2010) reported isolation, characterization and comparative study on
podophyllotoxin and related glycosides of *P. hexandrum*. HPLC, column and thin
layer chromatography guided studies led to the isolation of seven different
compounds in methanolic extracts of *P. hexandrum*. The isolated compounds were
analyzed using LC-MS and High Performance Liquid Chromatography (HPLC)
studies interfaced to mass spectroscopy. Isolated compounds were used successfully
as chemical markers for the comparison of the twelve different accessions of
*Podophyllum*. They have also shown that the variation of chemical composition in *P.
hexandrum* agree well with their botanical phylogeny as revealed by genetic
phylogeny. HPLC analysis also revealed development of valuable chemotypes
containing higher concentration of isolated marker compounds.

Enhanced production of podophyllotoxin by co-culture of transformed *Linum album*
cells with plant growth-promoting fungi has been given by Baldi et al (2010). To
investigate the plant growth-promoting effect and stimulation of lignan bio -synthesis,
the effect of culture filtrates/live co-culture of two arbuscular mycorrhizae-like fungi,
*Piriformospora indica* and *Sebacina vermifera*, on growth of *Linum album* cells and
on production of podophyllotoxin (PTOX) and 6-methoxypodophyllotoxin (6-MPT)
was studied. The culture filtrates of both the fungi exhibited a positive effect on
product formation. For co-culture experiments, both fungi were individually co-
cultivated at different concentrations with *L. album* in suspension cultures for different time periods. This resulted in significant enhancement of PTOX and 6-MPT content in the plant cells. The activity of phenylalanine ammonia lyase (PAL) was observed to be related to the lignan accumulation, indicating its role as the key enzyme of the phenylpropanoid pathway. The study resulted in total lignan (PTOX and 6-MPT) production of 745.6 mg/l with a very high PT productivity of 52.4 mg.

Chakraborty et al., (2010) developed an efficient protocol for *in vitro* regeneration of *P. hexandrum*. Rhizome explants were used to develop a protocol for *in vitro* plantlet regeneration, through direct organogenesis. Highest rate of multiple shoot formation was noted in MS medium supplemented with 11.2 µM IAA within three months. A synergistic effect of 2.68 µM NAA and 11.1 µM BAP was second best. The ½ MS liquid medium with 100 µM IBA was most suitable for rooting of shoots. Leaf explants resulted in callus formation only. Leaf regenerated calli showed the presence of podophyllotoxin in HPLC analysis.

Sharma et al., (2010) studies storage-dependent changes in dormancy and germination of Himalayan Mayapple (*Podophyllum hexandrum* Royle) seeds and their response to gibberellic acid. The storage-dependent changes in viability and dormancy/germination status of seeds of a Western population of Himalayan Mayapple have been monitored with particular reference to the changes in seed responsiveness to GA3. Shortly after harvest (3 months), seeds exhibited dormancy that was marginally overcome after storage for 30 months. GA3 treatment was effective in overcoming the dormancy, particularly when concentrated H2SO4 was used. The responsiveness of the seeds to GA3, however, decreased with the storage period. A subset of seeds that failed to germinate under a GA3-H2SO4-GA3 treatment eventually deteriorated. GA3-induced enhancement of the activities of α-amylase and dehydrogenases (2,3,5 triphenyltetrazolium chloride (TTC) reduction) corresponded to germination improvement in seeds stored for a short duration, but not in seeds stored for long period of time. Lipid peroxidation in seeds did not change owing to storage or GA3 treatment, whereas catalase activity tended to decline marginally. Seed phenolic contents were not involved in the seed germination behaviour.

Ionkova et al., (2010) studied production of podophyllotoxin in *Linum linearifolium* *in vitro* cultures. For the first time, callus and suspension cultures of *Linum*
linearifolium were initiated. Podophyllotoxin (PTOX), a strong antitumor precursor, was isolated from the calli and suspension, as a main lignan besides smaller amount of 6-methoxypodophyllotoxin (6MPTOX).

Elfahmi et al., (2010) studied reduced coniferin and enhanced 6-methoxypodophyllotoxin production in Linum flavum cell cultures. Treatment of cell suspension cultures of Linum flavum L. with Na₂EDTA reduced the coniferin and enhanced the 6-methoxypodophyllotoxin (6-MPT) production in a concentration-dependent way, in a range of 0.1–5 mM. On day 14 after treatment with Na₂EDTA, an inhibition of the coniferin production up to 88% was found. The maximum enhancement of the 6-MPT production was 400% on day 7 after treatment with 5 mM Na₂EDTA. The reduction in coniferin accumulation in the suspension cultures correlated with inhibition of coniferyl alcohol glucosyltransferase (CAGT) activity as determined in cell homogenates. On day 14 after treatment with 2 and 5 mM Na₂EDTA, the CAGT activity was inhibited up to > 89%. The inhibitory effect of Na₂EDTA on CAGT was also shown in a partially purified enzyme preparation. Several metal ions and the elicitors nigeran and salicylic acid had no significant effect on the production of coniferin and 6-MPT.

Nagar et al., (2011) studied different mode of action podophyllotoxin and their glycosidic derivatives (Etoposide and Teniposide) have. Podophyllotoxin inhibit the microtubules but its glycosidic derivatives like Etoposide and Teniposide inhibit DNA topoisomerase II by stabilising the covalent topo II- DNA cleavable complex. So, glycosidic derivatives of podophyllotoxin reduce the side effect.

Arumugam and Bhojwani, (2011) studied somatic embryogenesis in tissue cultures of P. hexandrum. The callus derived from zygotic embryos on Murashige and Skoog medium containing 2 M BA and 0.5 M IAA differentiated globular embryos. On this medium the globular embryos continued to multiply but failed to mature. Further development of the embryos occurred if the sucrose level in the basal medium was raised to 6% or the medium was supplemented with 1–10 M NAA. Light and temperatures higher than 25°C suppressed embryogenesis. Embryogenic potential of the callus has been maintained for over 20 months through subcultures. The somatic embryos developed into plantlets on the basal medium.
Renouard et al., (2011) studied podophyllotoxin and deoxypodophyllotoxin in *Juniperus bermudiana* and 12 other *Juniperus* species with respect to optimization of extraction, method validation, and quantification. *Juniperus bermudiana* was used for the development and validation of an extraction protocol for podophyllotoxin and deoxypodophyllotoxin allowing extraction yields of up to 22.6 mg/g DW of podophyllotoxin and 4.4 mg/g DW deoxypodophyllotoxin, the highest values found in leaf extract of *Juniperus*. The optimized extraction protocol and HPLC separation from DAD or MS detections were established and validated to investigate podophyllotoxin and deoxypodophyllotoxin contents in aerial parts of 12 other *Juniperus* species. This efficient protocol allows effective extraction of podophyllotoxin and deoxypodophyllotoxin from aerial parts of *Juniperus* species, which could therefore constitute interesting alternative sources of these valuable metabolites.

Kusari et al., (2011) studied chemometric evaluation of the anti-cancer pro-drug podophyllotoxin and potential therapeutic analogues in *Juniperus* and *Podophyllum* species. The individual and holistic metabolite profiles and chemometrically correlated the phytochemical loads between various species (intraspecific), organic and aqueous extracts, and populations of the same species from different locations, different species from same location, different species from different locations and infrageneric populations from same and different locations. Multivariate analysis revealed *Juniperus x-media* Pfitzeriana as a suitable alternative to *Podophyllum hexandrum* for commercial exploitation. A significant positive correlation of podophyllotoxone with both podophyllotoxin and demethylpodophyllotoxin, and a negative correlation of podophyllotoxin with both deoxypodophyllotoxin and demethylpodophyllotoxin (intraspecific among *Podophyllum*), were observed by Kruskal's multidimensional scaling and corroborated by principal component analysis, indicating probable similarity and/or difference between the biosynthetic pathways, and synergistic and/or antagonistic principles, respectively. Finally, linear discriminant analysis and hierarchical agglomerative cluster analysis revealed considerable infrageneric and intraspecific variability in secondary compound spectra and load of the different populations under study. Such holistic studies of plants and their therapeutic metabolites ought to assist in selecting plants, geographical areas and
environmental conditions for bioprospecting and global-scale phytochemical and phylogenetic diversity studies in the future.

Hendrawati et al., (2011) studied identification of lignans and related compounds in *Anthriscus sylvestris* by LC–ESI-MS/MS and LC-SPE–NMR. The latter serves as a starting compound for the production of cytostatic drugs like etoposide. A better insight into the occurrence of deoxypodophyllotoxin combined with detailed knowledge of its biosynthetic pathway(s) may help to develop alternative sources for podophyllotoxin. Using HPLC combined with electrospray tandem mass spectrometry and NMR spectroscopy techniques, they found nine lignans and five related structures in roots of *Anthriscus sylvestris* (L.) Hoffm. (Apiaceae), a common wild plant in temperate regions of the world. Podophyllotoxone, deoxypodophyllotoxin, yatein, anhydropodorhizol, 1-(3′-methoxy-4′,5′-methylenedioxyphenyl)1-methoxy-2-propene, and 2-butenolic acid, 2-methyl-4-[(2Z)-2-methyl-1-oxo-2-buten-1-yl]oxy]-, (2E)-3-(7-methoxy-1,3-benzodioxol-5-yl)-2-propen-1-yl ester, (2Z)- were the major compounds. α-Peltatin, podophyllotoxin, β-peltatin, isopicropodophyllone, β-peltatin-a-methylether, (Z)-2-angeloyloxymethyl-2-butenolic acid, anthriscinol methylether, and anthriscrusin were present in lower concentrations. α-Peltatin, β-peltatin, isopicropodophyllone, podophyllotoxone, and β-peltatin-a-methylether have not been previously reported to be present in *A. sylvestris*. Based on our findings we propose a hypothetical biosynthetic pathway of aryltetralin lignans in *A. sylvestris*.

Kitchlu et al., (2011) studied *Podophyllum* lignans array of *P. hexandrum* populations from semi-desert alpine region of Zanskar valley in Himalayas. Further, ontogenetic and morphogenetic variations of *Podophyllum* lignan contents were studied to investigate dynamics of accumulation of these compounds. Representative collections from three locations viz., Panikhar, Padam and Tangoli located in Trans Himalayan semi-desert region of Zanskar valley were harvested at three stages (dormancy, active growth and maturity). Plants were dissected into root, rhizome and rhizome-buds, dried separately and assayed for *Podophyllum* lignan contents by high performance liquid chromatography.

Avula et al., (2011) studied rapid analysis of lignans from leaves of *Podophyllum peltatum* samples using UPLC-UV-MS. The chromatographic separation was achieved using a reversed-phase C18 column with a mobile phase of water and
acetonitrile, both containing 0.05% formic acid. Analyses of *P. peltatum* leaves collected from different colonies within a single site indicated a significant variation in 4′-O-demethylpodophyllotoxin, α-peltatin, podophyllotoxin and β-peltatin content. Within 3.0 min four main lignans could be separated with detection limits of 0.1, 0.3 and 0.2 g/mL, respectively. 4′-O-demethylpodophyllotoxin and α-peltatin appeared most prominently among the lignans obtained. The podophyllotoxin content was found in the range of 0.004–0.77% from 16 samples collected from 6 colonies within the same site. The content of podophyllotoxin is directly proportional to the content of 4′-O-demethylpodophyllotoxin and inversely proportional to α-peltatin and β-peltatin content.

Majumdar, (2012) studied influence of an indirect precursor on podophyllotoxin accumulation in cell suspension cultures of *P. hexandrum*. In liquid P1 medium [half strength B5 basal medium supplemented with 1% (w/v) sucrose, 2,4-D (1 mg/l), GA3 (1 mg/l) and BAP (0.1 mg/l)]. The effect of different concentrations (100, 250 and 500 mg/l) of tryptophan, an indirect precursor of lignan biosynthesis, was studied on cell suspension cultures. Cultures accumulated 2.7 times more podophyllotoxin than untreated control cultures when treated with 250 mg/l tryptophan. Biomass yield was, however not improved following the addition of tryptophan.

Wani et al., (2012) developed the method of TLC profiling. Phytochemical analysis revealed the presence of diverse groups of phyto-constituents in two different rhizome extracts (aqueous and methanolic). Chemical constituents have shown different Rf values 0.363, 0.60, 0.727 in methanolic extract but in aqueous extract only one spot and shows Rf value 0.410.

Yousefzadi et al., (2012) conducted series of experiments was carried out using ordinary fluorescent lamps to study the influence of light irradiation on growth and PTOX accumulation in *Linum album* cell cultures by varying the type of light and periods of exposure. The biosynthesis of PTOX was variably affected according to the quality of light. The enhancing effects of red light on PTOX production was correlated with increased activities of the enzyme phenylalanine ammonia-lyase (PAL), and the expression of some key genes involved in the biosynthesis of this compound, including the PAL gene itself and the cinnamoyl-CoA reductase (CCR)
gene. Blue light was found to have similar effects but mainly on the expression level of CCR and pinoresinol lariciresinol reductase (PLR) genes.

Sharma, (2013) has developed a simple, sensitive, selective and reliable HPLC method based on photo diode array detector (PDA) for the simultaneous determination of an important compound (podophyllotoxin) in leaves and rhizomes parts of four different populations of *P. hexandrum* from higher altitude Himalayas. The analysis was carried out on a RP-18e (LICrosphere, 5 μm, 250 x 4.0 mm) column, with isocratic elution of acetonitrile: water (40:60, v/v). The method was validated for accuracy, precision, limit of detection and quantification. The regression equation revealed a good linear relationship (*r*² = 0.9999 for podophyllotoxin) within test ranges. The limit of detection and limit of quantification for analyte in PDA was 50 and 110 g, respectively. The method showed good reproducibility for the quantification of podophyllotoxin in *P. hexandrum*.

Veloz et al., (2013) reported production of podophyllotoxin from roots and plantlets of *Hyptis suaveolens* cultivated in vitro. Explants of *H. suaveolens* were cultivated in Murashige and Skoog (MS) medium supplemented with different concentrations of the phytohormones 6-benzylaminopurine (6-BAP), 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA) and kinetin (Kin), in order to induce the production of podophyllotoxin. Root cultures without hormones were also established and the quantification of PTOX was performed by HPLC analysis. The presence of growth regulators during in vitro cultivation of *H. suaveolens*, provoked morphological variations in explants, and induced the accumulation of different levels of PTOX. Roots grown without phytohormones accumulated PTOX at 0.013% dry weight (DW), while in three of the callus cultures cell lines growing together with roots, PTOX accumulated at concentrations of 0.003, 0.005 and 0.006% DW when NAA was combined with either Kin or BAP. In wild plant material PTOX was present in trace amounts in the aerial parts, while in the roots it was found at 0.005% DW. This study demonstrated that although it is possible to obtain PTOX in a variety of in vitro cultures of *H. suaveolens*, in vitro roots grown without the addition of growth regulators were better producers of PTOX.

Cantrell et al., (2013) have studied the podophyllotoxin and essential oil profile of *Juniperus* and other collected from different location in the U.S. they have categorised
J. virginiana into four groups with respect to podophyllotoxin, essential oil contents and other related products. They further classified J. virginiana accessions into 10 chemotypes on the basis of essential oil composition. The availability of various chemotypes offers an opportunity for the development of cultivars for commercial production of podophyllotoxin and essential oil with specific compositional profile to meet the market requirements.

Pandey et al., (2013) reported podophyllotoxin content in leaves and stems of P. hexandrum from Indian Himalayan region. The source of podophyllotoxin from leaves and stems of three morphological variants of P. hexandrum with one leaf (1 L), two leaves (2 L) and three leaves (3 L), collected from seven populations (2800 to 3600 m) of Uttarakhand state in Indian central Himalaya. In general, podophyllotoxin content (on % dry wt.) varied significantly ($p < 0.05$) between the morphological variants, in both leaf and stems. The content of leaf ranged from 0.001 to 0.599%; maximum (0.599%) podophyllotoxin in 3 L (Dodital), and minimum (0.001%) in 1 L and 3 L (Kedarnath) plants were detected. The mean podophyllotoxin content (population wise) was significantly ($p < 0.05$) higher in Dodital population, with maximum value (0.229%) in Dodital and minimum (0.003%) in Kedarnath. The levels in stem samples were found in the range of 0.001 to 0.596%, similar to those found in the leaves. While maximum amount (0.596%) was estimated in 3 L plants from Dodital, the minimum (0.001%) level was found in 1 L (Kedarnath), 2 L (Garur Chatti) and 3 L (Kedarnath) plants. The mean podophyllotoxin content (population wise) was significantly ($p < 0.05$) higher in Dodital, Dayara and Bharnala populations, with maximum value (0.234%) in Dodital. Both the leaves and stems of 3 L plants (from Dodital) contained maximum levels of podophyllotoxin.