CHAPTER 1

INTRODUCTION
Herbal drug formulations have been an integral part of Ayurvedic system of medicine for centuries. In the last part of twentieth century, Western Nations realized the importance of herbal medicine as the one that possesses maximum health benefits with minimum adverse effects [Sharma, 2008; Tovar, 2009]. Herbal drugs are prepared from plants containing active constituents of medicinal importance [Banerjee & Mitra, 2011]. The herbal medicinal products are dietary supplements that are taken to improve health and are sold as tablets, capsules, powders, teas, extracts and fresh or dried plants. The use of herbal medicine has been on increase in many developing and industrialized countries [Furnharm, 1996; Ernst, 2003]. It is known that between 65 and 80% of the world's population use herbal medicines as their primary form of health care [Eisenberg et al., 1998]. Many drugs used in conventional medicine were originally derived from plants. Salicylic acid is a precursor of aspirin that was originally derived from white willow bark and the meadowsweet plant (Filipendula ulmaria (L.) Maxim.) [Raskin, 1992]. Quinine and Artemisinin are antimalarial drugs derived from Cinchona pubescens Vahl bark and Artemisia annua L. plant, respectively [Covello, 2008]. Vincristine is an anticancer drug derived from periwinkle (Catharanthus rosae Linn. G. Donn.) [Arcamone et al., 1980]. Morphine, codeine, and paregoric, derived from the opium poppy (Papaver somniferum L.), are used in the treatment of diarrhea and pain relief [Elhardallou, 2011]. Digitoxin is a cardiac glycoside derived from foxglove plant (Digitalis purpurea L.) an herb in use since 1775 [Hollman, 1985]. In folklore medicine in Nigeria Rauwolfia vomitoria is used for treating hypertension, stroke, insomnia and convulsion [Amole et al., 2009] and Ocimum gratissimum L. is used for treating diarrheal diseases [Ilori et al., 1996]. The herbal drugs have thus been in usage for thousands of years due to their low/minimum cost, potency and efficiency, enhanced tolerance, fewer side-effects, complete accessibility and recyclable [Maiti et al., 2011].

With an ever-increasing global demand for herbal medicine, there is not only a demand for large quantity of raw material of medicinal plants, but also of appropriate
quality where active principles are available in desired concentrations [Shahzad & Saeed, 2013]. At present 95% collection of medicinal plants is from the wild. Many studies have confirmed that pharmaceutical companies are responsible for inefficient, imperfect, informal, and opportunistic marketing of medicinal plants [Verma et al., 2012]. As a result, the raw-material supply situation is shaky, unsustainable, and exploitative. There is a vast, secretive, and largely unregulated trade in medicinal plants, mainly from the wild, which continues to grow dramatically in the absence of serious policy attention with environmental planning. Confusion also exists in the identification of plant materials where the origin of a particular drug is assigned to more than one plant, sometimes having vastly different morphological and taxonomical characters; therefore, adulteration is common in such cases [Kala, 2000; Kala, 2003; Schippmann et al., 2006]. The medicinal and aromatic plants and their wild populations are facing serious threats due to (1) the intensive and increasing commercial collection, often concentrated in few areas, (2) the largely unmonitored trade, (3) destructive harvesting techniques, (4) trade structure changes in countries and (4) global habitat loss and alteration [Lange, 2004].

The quantity and efficacy of a herbal drug formulation is influenced due to the presence of desired contents and combination of major chemical constituents, which in most of the case are secondary metabolites. Plant secondary metabolites are highly diverse in their chemical structures and are classified into three major groups: terpenoids, phenolic compounds, and nitrogen containing compounds. More than 36,000 terpenoids, 12,000 alkaloids, and 10,000 flavonoids have been found, although this represents only a fraction of what exists in nature [Chen et al., 2007; Fang, 2011].

The biosynthesis and accumulation of medicinally important metabolites occur in different tissues and organs of plants and is largely influenced by the developmental stage of a particular organ/tissue as well as external stimuli. These factors play an important role, as the uniformity in the selection of plant material is essential for the preparation of herbal drug formulation. It is important to realize that different batches of the same herbal
ingredient may differ in quality due to a number of factors. Active constituents usually vary between plant parts and it is not uncommon for herbal ingredients to be adulterated with parts of the plants not normally utilized. The quality of herbal ingredients can be affected by environmental factors like climate, altitude and other conditions under which it was cultivated. For some herbs the optimum time of harvesting should be specified, as it is known that the concentrations of constituents in a plant can vary during the growing cycle or even during the course of a day [Kunle et al., 2012]. For example the accumulation of hypericin and hyperforin occurs in leaves and inflorescence of *Hypericum perforatum*, respectively [Kowalski & Wolski, 2006]. The accumulation of withanolide occurs in shoot tips and leaves of *Withania somnifera* [Praveen et al., 2010]. The amounts of vanillin, glucovanillin and other metabolites were higher in the older pods compared to younger pods of *Vanilla planifolia* [Palama et al., 2009]. Similarly the biosynthesis of rutin is influenced by the growth and developmental stages of *Fagopyrum* species [Gupta et al., 2011]. Increase in podophyllotoxin content was reported in the rhizomes of different age groups of *Podophyllum hexandrum Royle* [Pandey et al., 2007]. Similarly the amount of berberine increased in the roots and rhizomes of 5-year-old *Berberis darwini* compared to 3 year old plants [Cromwell, 1933]. The optimization of above mentioned factors would, therefore, be helpful for the farmers, collectors of herbal raw material, researchers and herbal drug manufacturers in obtaining best quality plant material at a particular time when the active principles remain at their maximum concentration.

*Picrodiza kurroa* Royle ex. Benth. is an important medicinal herb (Family: Scrophulariaceae) mainly found in the Western Himalayan region, between 3000-5000 m elevation [Hooker et al., 1885; Chopra et al., 1934; Blatter, 1984; Jain, 1996; Agrawal, 2003]. It is valued as a hepato-protective, immunomodulator, anti- periodic, stomachic, anti-oxidant, anthelmintic, anti-inflammatory, cardio-tonic, laxative, carminative, expectorant, etc. [Chopra et al., 1934; Uphof, 1959; Singh et al., 1983; Kapoor, 1990; Kapahi et al., 1993; Bhatt & Bhatt, 1996; Gaddipati et al., 1999; Prajapati et al., 2003].
Aqueous rhizome extracts of *P. kurroa* have shown hepatoprotective and antioxidant properties on CCl₄ induced liver toxicity in albino rats [Vinothkumar *et al*., 2010], antioxidant and anti-neoplastic activities [Rajkumar *et al*., 2011] and a iridoid glycoside isolated from the roots was an effective immuno modulator specifically to improve macrophage function during infections [Sidiq *et al*., 2011]. Recently rhizome extracts of *P. kurroa* have also shown to possess anti-malarial activity [Singh & Banyal, 2011; Irshad *et al*., 2011]. Standardized iridoid fractions of *P. kurroa*, e.g. kutkin and Picroliv consist of glucosides, picroside-I (P-I) and kutkoside also known as picroside-II (P-II) in a ratio of 1:2 and other minor glycosides [Singh & Rastogi, 1972; Ansari *et al*., 1988]. Picroliv, which was launched as a herbal drug formulation, is prepared from a standardized iridoid fraction containing 60% of P-I and Kutkoside in a 1:1.5 ratio [Dwivedi *et al*., 1989; Ansari *et al*., 1991; Dhawan, 1995]. Picroliv has also been shown to have immunostimulating effect in hamsters and helps to prevent infections [Puri *et al*., 1992; Gupta *et al*., 2006]. There are several other commercially available drug formulations containing P-I and P-II, e.g. Katuki, Zandu Pharma works Ltd. (Mumbai, India) having P-I (1.29%) and P-II (1.16%), *(Table 1.1)* [Bhandari *et al*., 2009].
<table>
<thead>
<tr>
<th>Sample</th>
<th>P-I (%)</th>
<th>P-II (%)</th>
<th>Total picrosides content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Katuki, Zandu Pharma Works Ltd. (Mumbai, India)</td>
<td>1.29</td>
<td>1.16</td>
<td>2.45</td>
</tr>
<tr>
<td>Arogya, Zandu Pharma Works Ltd. (Vapi, GJ, India)</td>
<td>1.01</td>
<td>0.55</td>
<td>1.56</td>
</tr>
<tr>
<td>Kutaki, Tansukh Herbal Pvt. Ltd. (Lucknow, UP, India)</td>
<td>4.17</td>
<td>3.25</td>
<td>7.42</td>
</tr>
<tr>
<td>Livocare, Dindayal Aushdi Pvt. Ltd. (Gwalior, MP, India)</td>
<td>0.06</td>
<td>0.14</td>
<td>0.20</td>
</tr>
<tr>
<td>Livomap, Maharishi Ayurveda Pvt. Ltd. (New Delhi, India)</td>
<td>0.12</td>
<td>0.06</td>
<td>0.18</td>
</tr>
<tr>
<td>Livomyn, Charka Pharma Pvt. Ltd. (Mumbai, India)</td>
<td>0.07</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>Livplus, BACFO Pharmaceuticals Ltd. (Noida, UP, India)</td>
<td>0.22</td>
<td>0.49</td>
<td>0.71</td>
</tr>
<tr>
<td>Pravekliv, Pravek Kalp Herbal products Pvt. Ltd. (Noida, UP, India)</td>
<td>0.15</td>
<td>0.12</td>
<td>0.27</td>
</tr>
</tbody>
</table>
The P-I and P-II are, therefore, two major chemical constituents present in *P. kurroa* (Figure 1.1) that have therapeutic importance in several herbal drug formulations [Dutt et al., 2004]. The proper concentration and ratio of P-I and P-II are, therefore, important in determining the quality and efficacy of *P. kurroa* based herbal drug formulations.

Figure 1.1. Chemical structure of Picroside-I and Picroside-II

The *P. kurroa* propagates vegetatively through stolons, which initially emerge as a young bud, grow to a mature stolon and then eventually into a rhizome with independent shoots and roots [Raina et al., 2010] (Figure 1.2). The *P. kurroa* plants are uprooted from their natural habitat for obtaining mature rhizomes, however, along with all other young stolons, the rhizomes also get uprooted, thereby, disrupting the natural propagation of *P. kurroa* (Figure 1.2). The increasing national and international demand for *P. kurroa* raw material coupled with its reckless collection from the natural habitat and limited cultivation has made it a critically endangered plant species [Nayar & Sastri, 1990; Rai et al., 2000]. The plant produces relatively small amounts of picrosides in the rhizomes, that too after their growth and maturity until 3-4 years, which therefore necessitates the development of intervention strategies so as to increase the yield of picrosides.
The biology of biosynthesis of P-I and P-II has been poorly understood with only the P-I detected in shoots, whereas both accumulate in rhizomes [Sood & Chauhan, 2010]. However, what determines the biosynthesis of P-I in shoots and then why and how both (P-I and P-II) accumulate in rhizomes is not known.

**Figure 1.2 Mature P. kurroa plant (~3 years old)**

The biosynthesis and accumulation of P-I and P-II occur in *P. kurroa* at high altitudes (1900–4500m) and that too during a particular time of a season, which complicates the process of understanding biology of their biosynthesis. Understanding the dynamics of P-I and P-II contents in different growth and developmental stages of *P. kurroa* is, therefore, of paramount importance not only for gaining insights into the biology of their biosynthesis but also regulating the quality of herbal drug formulations prepared from *P. kurroa* so that agro-based technologies can be optimized for the commercial cultivation and production of uniform plant material. Recent studies have suggested that the leaves, rather than the
underground parts such as rhizome and roots, might be used as a plant material for the preparation of herbal drug formulations from *P. kurroa* [Katoch *et al.*, 2011]. On the other hand, leaves are known to contain only P-I not P-II, which, therefore, necessitate thorough understanding of the biology of P-I and P-II biosynthesis and accumulation in different growth stages of *P. kurroa* so that the quality of herbal drug formulations is not compromised [Sood & Chauhan, 2010]. The effect of altitude has also been suggested as an important factor influencing P-I and P-II contents in *P. kurroa*, P-I and P-II was highest in populations collected from Sonemarg (2,740m a.s.l.) followed by Tangmarg (2,690m a.s.l.), suggesting that picroside accumulation is directly correlated with altitudinal change [Katoch *et al.*, 2011]. However, collections of *P. kurroa* made from different altitudes would not only reflect the influence of environment at a particular altitude on the biosynthesis and accumulation of P-I and P-II, but also the collections can be genetically different strains. Therefore, the effect of altitude or other environmental factors on biosynthesis and accumulation of picrosides can be best understood if same strain(s) of *P. kurroa* is grown at different altitudes and then quantified for the P-I and P-II contents in tissues of different growth and developmental stages. The current study has quantified P-I and P-II contents in field grown plants of *P. kurroa* vis-à-vis different growth and developmental stages. The effect of altitude on P-I and P-II contents in different organs and developmental stages of the same strain of *P. kurroa* was also investigated. Any genetic intervention for enhancing or altering the contents of P-I and P-II in *P. kurroa* would require understanding the biosynthetic pathway(s) contributing to their biosynthesis. P-I and P-II are iridoid derivatives of monoterpenes [Smit, 2000]. The isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP) condense to form geranyl diphosphate (GPP), the precursors of monoterpenes [Dudareva *et al.*, 2004]. The biosynthesis and accumulation of terpenoids is controlled at the molecular level by structural and regulatory genes in different plant species [Olofsson *et al.*, 2011]. The nonmevalonate (MEP) and mevalonate (MVA) biosynthetic pathways lead to the biosynthesis
of DMAPP (dimethyl allyl pyrophosphate) and IPP (isopentenyl pyrophosphate), which are the building blocks of GPP the starting material for the biosynthesis of P-I and P-II (Figure 1.3). However, there are reports where either MVA or MEP pathway contributes to the biosynthesis of GPP such as some monoterpenes in strawberry (Fragaria spp.) have an exclusive MVA origin [Hampel et al., 2006]. The sesquiterpenes from mint (Mentha spp.) essential oils are derived from MEP pathway [McCaskill & Croteau, 1995; Lange et al., 2000; Dudareva et al., 2005]. Sesquiterpene precursors in carrot (Daucus carota), chamomile (Matricaria chamomilla) and Solidago are derived from both the MEP and MVA pathways [Adam et al., 1999; Steliopoulos et al., 2002; Hampel et al., 2005].

Two different biosynthetic routes can synthesize both DMAPP and IPP: 1- deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4- phosphate (DXP/MEP) pathway in plastids and/or the classical mevalonate (MVA) pathway in cytoplasm [Kai et al., 2006]. Plants use both MEP and MVA pathways for isoprenoid biosynthesis, although they are localized in different compartments [Lange et al., 2000; Rodriguez & Boronat, 2002].
Non-Mevalonate pathway

Pyruvate + glyceraldehyde-3-phosphate  \(\rightarrow\)  DXPS
1-deoxy-D-xylulose-5-P  \(\rightarrow\)  DXPR
2-C-methyl-D-erythritol  \(\rightarrow\)  ISPD
4-(CDP)-2-methyl-D-erythritol  \(\rightarrow\)  ISPE
4-(CDP)-2-methyl-D-erythritol-2-P  \(\rightarrow\)  MECP3
2-C-methyl-D-erythritol-2,4 cyclo-PP  \(\rightarrow\)  HDS
1-hydroxy-2-methyl-2-(E)-butenyl-4-PP  \(\rightarrow\)  ISP3H
Dimethylallyl pyrophosphate  \(\leftrightarrow\)  IPPI  \(\rightarrow\)  Isopentenyl pyrophosphate

Mevalonate pathway

Acetyl-Co-A  \(\rightarrow\)  ACTH
Acetoacetyl-Co-A  \(\rightarrow\)  HMGS
3-HMG-Co-A  \(\rightarrow\)  HMGR
Mevalonate  \(\rightarrow\)  MVK
Mevalonate phosphate  \(\rightarrow\)  PMK
Mevalonate pyrophosphate  \(\rightarrow\)  MVPP

Dimethylallyl pyrophosphate  \(\rightarrow\)  GDP3  \(\rightarrow\)  Geranyl pyrophosphate
Cinnamic acid/Vanillin acid  \(\rightarrow\)  Iridoid moiety  \(\leftarrow\)  Glucose

Picronides (I & II)

Figure 1.3 Schematic pathway for picroside biosynthesis; Adapted from Mahmoud & Croteau [2002].

The MEP pathway is essential for plastidal isoprenoid biosynthesis in plants [Rodriguez et al., 2004]. The MEP pathway starts with the formation of 1-deoxy-D-xylulose 5 phosphate (DXP) from D-glyceraldehyde 3-phosphate and pyruvate by the catalytic action of a 1-deoxy-D-xylulose 5-phosphate synthase [Sprenger et al., 1997, Bouvier et al., 1998] and then, DXP is converted into IPP by a series of enzymes. Total 15 genes, 8 of MEP pathway,
6 of MVA pathway, and IPPI convert DMAPP or IPP into GPP in monoterpenoids biosynthesis [Wise & Croteau, 1998]. Out of 15 genes, sequences for 10 genes were available in the GenBank but not for the remaining 5 genes at the take up of this study. The expression of 2 genes \textit{hmgr} and \textit{dxs} was shown to be up regulated in \textit{P. kurroa} vis-à-vis picrosides biosynthesis in plants exposed to different light and temperature regimes [Kawoosa \textit{et al.}, 2010]. Both these genes are reported as rate limiting steps in MVA and MEP pathways in different plant species, which was considered as an assumption that the same genes might be limiting the biosynthesis of P-I and P-II in \textit{P. kurroa} [Kawoosa \textit{et al.}, 2010]. However, ascertaining the role of particular gene(s) in P-I or/and P-II biosynthesis/contents would require expression analysis of all 15 genes of MEP and MVA pathways vis-à-vis P-I and P-II contents in different tissues and organs of \textit{P. kurroa}. The current study undertook the cloning of remaining 5 genes, ISP, MFCPS, HDS, HMGS and PMK of MEP and MVA pathways in \textit{P. kurroa} and checked the expression profile of all 15 genes of MEP and MVA pathways in different tissues in relation to P-I and P-II contents.

Keeping in view the medicinal importance of \textit{P. kurroa}, lack of information on the biology of P-I and P-II biosynthesis and accumulation and virtually no information on the molecular basis of picroside biosynthesis in \textit{P. kurroa}, necessitated taking current study with the following objectives:

**OBJECTIVES**

- Detection and quantification of Picroside-I & Picroside-II in different tissues and growth/developmental stages of \textit{P. kurroa}
- Expression analysis of MVA and MEP pathway genes vis-à-vis Picroside-I and Picroside-II contents in different growth stages of \textit{P. kurroa}