CHAPTER 1: Introduction

*Picrorhiza kurrooa* [P. kurrooa Royle ex Bentham; family *Scrophulariaceae* (2n=34)] is a medicinally important, perennial herb with stout and creeping rhizome (Fig. 1.1). The plant generally occurs at an elevation of ≥ 3000 amsl in the moist rocky habitats with well drained soil in India, Nepal, Bhutan, China and Myanmar. In India, *P. kurrooa* is mainly distributed in the north-west, north-east, central and trans-himalayan biogeographic zones (Rai & Sharma 2002, Haridasan *et al.* 2002). In Himachal Pradesh, the plant is distributed in Great Himalayan National Park, Dhauladhar WLS, Kugti WLS, regions of Lahaул-Spiti and alpine regions of Mandi, Kullu, Chamba, and Kinnaur districts (Uniyal *et al.* 2006; Rawat, 2008).

![Image of Picrorhiza kurrooa](image)

**Fig. 1.1:** A. *Picrorhiza kurrooa* growing at Rohtang Pass (4,000 m altitude, 32°23' N, 77°15' E, India) in Kullu district of Himachal Pradesh, India. B. Different parts of *P. kurrooa* are shown by arrow.
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The medicinal properties of *P. kurrooa* are attributed to iridoid-glycosides, commonly known as picrosides. Picrosides are reported to be present in rhizome tissue (Verma *et al.*, 2009; Ansari *et al.*, 1988) and hence the entire plant is uprooted for trading purpose. Extensive harvesting and lack of organized cultivation has threatened its status in the wild. The plant is listed as vulnerable species in Red Data Book (Nayar and Shastri 1990) and subsequently under the ‘endangered’ species category, as per Conservation Assessment and Management Planning (CAMP) workshop (Ved *et al.* 2003). Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed *P. kurrooa* in its **annexure II** that restricts its trade across nation’s territory (CITES, 2000).

![Chemical structure of Picroside I and Picroside II](image)

**Fig. 1.2: Chemical structure of Picroside I and Picroside II.**

“Picrosides” is a collective term used for standardized iridoids fraction of *P. kurrooa*, called kutkin and picroliv. Kutkin is obtained by crystallization of iridoids fraction and consists of the glycosides, P-I and kutkoside in a ratio of 1:2 and other minor glycosides (Singh and Rastogi, 1972, Ansari *et al.*, 1988). Picroliv is similar to kutkin but is a less purified fraction, containing about 60% of an equal mixture of picroside I and kutkoside (Dwivedi *et al.*, 1989). More recent publications described Picroliv as a standardized iridoid fraction containing 60% of this mixture in a 1:15 ratio (Dhawan, 1995). Other glycosides reported from *P. kurrooa* are P-II, picroside III, picroside IV and picroside V.

P-I and P-II are the major picrosides, which differ from each other by the attached functional group. In P-I, cinnamoyl group is attached to a sugar moiety, while in P-II vanilloyloth.
moiety is attached to 6th carbon of iridoid ring (Fig. 1.2) (Shah and Chauhan, 1990). Experiments have shown that picrosides possess hepatoprotective (Vaidya et al., 1996) and immunomodulatory (Gupta et al., 2006) activities. Picroliv, which is chiefly composed of P-I and P-II, is reported to have hepatocurative effect against alfatoxin B1 induced hepatotoxicity in rats (Rastogi et al., 2001). It also has immuno-stimulant activity against Leishmania donovani infection in hamsters (Puri et al., 1992). Activity of P-glycoprotein (P-gp), which is one of the xenobiotic transport proteins implicated in multidrug resistance in neoplastic tissues, are reported to be modulated by P-I and P-II (Najar et al., 2010). In cancer tissue, P-gp functions as a drug export pump that decreases intracellular concentrations of numerous chemotherapeutic agents (Gottesman et al., 1996). P-II has been reported to protect hepatocytes against injury and prevent hepatocytes from apoptosis (Gao and Zhou, 2005). Also, P-II has been reported to protect cells from hydrogen peroxide induced oxidative stress and is a potential therapeutic agent for treating nervous disorders (Cao et al., 2007).

1.1 Biosynthesis of Picrosides

Picrosides are monoterpenes with an iridane skeleton. Monoterpenes are derived from GDP. GDP is synthesized by sequential head to tail addition of IPP and its allelic isomer, DMAPP through MVA and MEP pathways. A preference for MVA versus MEP pathway depends upon the metabolites and the plant species under consideration (Sando et al., 2008). In general, biosynthesis of picrosides can be described in four phases:

1. synthesis of the terpenoid building units IPP and DMAPP
2. condensation of IPP and DMAPP by geranyl diphosphate synthase to form GDP
3. conversion of GDP to the iridoids
4. transformation of the iridoid parent skeleton into picrosides by addition of glucose and shikimate pathways derivative, respectively

Previously, MVA pathway of isoprenoid biosynthesis was considered to be the universal route for the production of IPP (Bochar et al., 1999). However, it is now established that plant cell produces IPP via two chemically and spatially distinct and independent pathways. In the cytosol, IPP is generated by acetyl-CoA via the MVA pathway, which supplies precursor for the biosynthesis of sesquiterpene (Newman and Chappell, 1999). In
plastids, MEP pathway produces IPP for the biosynthesis of monoterpene (Rohdich et al., 2001). In MVA pathway (Fig. 1.3), three molecules of acetyl-CoA condense to yield hmg-CoA, which is reduced by the enzyme hmgr to yield MVA. In the next two steps, MVA is phosphorylated twice by mvk and pmvk to form MVD, which in turn is decarboxylated to yield IPP and DMAPP.

MEP pathway starts by the transketolase-type of condensation of pyruvate with G-3P to yield DXP, and is followed by isomerization and reduction of DXP to MEP. The MEP, via cytidine-5-diphosphate derivative, undergoes phosphorylation at second carbon (C-2) followed by cyclization to yield 2-C-methyl-erythritol-2, 4-cyclo-diphosphate. Subsequently, reductive ring opening produces HMBPP, which via 4-hydroxy-DMAPP yields IPP and DMAPP (Lichtenthaler et al., 1997a).

IPP and its allylic isomer DMAPP is converted into GDP by the action of enzyme gdps. GDP after cyclisation yields iridoid; addition of glucose and cinnamate yields P-I, whereas addition of glucose and vanillate to this moiety yields P-II.

The compartmental separation of these two IPP biosynthetic pathways is not absolute, because at least one of the metabolites can be exchanged between the compartments (Arigoni et al., 1997). The extent of this crosstalk depends on the species as well as on the presence and concentration of exogenous precursors. Generally, crosstalk is assumed to be small in intact plants (<1%) under physiological conditions (Eisenreich et al., 2001). Higher values have been found in plant cell cultures in the presence of exogenous 1-deoxyxylulose or mevalonate. The nature of the metabolite(s) exchanged between the compartments and the regulation of the process remains to be established (Eisenreich et al., 2001).

Supply of GDP is critical in realizing the yield of isoprenoids (Nogués et al., 2006), therefore, studies on the regulation of gene expression associated with GDP biosynthesis is of immense significance. Molecular regulation of isoprenoid biosynthesis has been carried out in Catharanthus roseus (Arigoni et al., 1997), Antirrhinum majus (Dudareva et al., 2005) and Arabidopsis thaliana (Laule et al., 2003). However, no work has been done on the molecular aspects of picrosides biosynthesis in P. kurrooa.
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**MEP Pathway**

Pyruvate + Glyceraldehyde → 1-Deoxy-D-xylulose-5-P → 2-C-methyl-D-erythritol-4-P → 4-(CDP)-2-C-methyl-D-erythritol → 4-(CDP)-2-C-methyl-D-erythritol-2-P → 2-C-methyl-D-erythritol-2,4-cyclo-PP → 1-Hydroxy-2-methyl-2-E-buteryl-4-PP → Dimethylallyl pyrophosphate \[\leftrightarrow\] Geranyl dipiphosphate → Iridoid moiety

**MVA Pathway**

Acetyl coenzyme A → Acetoacetyl coenzyme A → 3-Hydroxy-3-methylglutaryl coenzyme A → Mevalonate → Mevalonate phosphate → Mevalonate pyrophosphate → Isopentenyl pyrophosphate

Fig. 1.3: Schematic pathway for picroside biosynthesis (adapted from Kawoosa et al., 2010). GDP can be derived from MVA or MEP pathway. GDP yields iridoid, which is converted into picroside in the presence of glucose and cinnamic acid or vanillic acid. Encircled numbers represent enzyme catalyzing the corresponding reaction step as follows: 1, 1-deoxy-D-xylulose-5-phosphate synthase; 2, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; 3, 2-C-methylerythritol 4-phosphate cytidyl transferase; 4, 4-(cytidine-5' -diphospho)-2-C-methylerythritol kinase; 5, 2-C-methylerythritol-2,4-cyclophosphate synthase; 6, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; 7, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase; 8, acetoacetyl CoA thiolase; 9, 3-hydroxy-3-methylglutaryl coenzyme A synthase; 10, 3-hydroxy-3-methylglutaryl coenzyme A reductase; 11, mevalonate kinase; 12, phosphomevalonate kinase; 13, mevalonate-5-pyrophosphate decarboxylase; 14, isopentenyl pyrophosphate isomerase; 15, geranyl dipiphosphate synthase; 16, caffeoyl-CoA methyltransferase; 17, phenylalanine ammonia lyase. Solid arrows indicate known steps, whereas dotted arrows indicate unknown steps.
Realizing the medicinal importance of the endangered species *P. kurrooa*, and that no molecular work has been done on this plant, the following objectives were laid down for the present thesis:

1. Cloning and characterization of the various genes involved in picrosides biosynthetic pathway
2. Spatio-temporal regulation of above genes of the pathway