CHAPTER 6: SUMMARY

*P. kurrooa* Royle ex Bentham [family; Scrophulariaceae (2n=34)] is a medicinally important, perennial herb with stout and creeping rhizome. In India, *P. kurrooa* is mainly distributed in the high altitudes of Himalaya. The medicinal properties of *P. kurrooa* have been attributed to iridoid-glycosides, commonly known as picrosides. Picrosides are reported to be present in rhizomatous 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 wild and is listed as ‘endangered’ species by International Union for Conservation of Nature and Natural Resources (Nayar and Sastri 1990).

P-I and P-II are major picrosides which are associated with several biological functions. These picrosides form major fraction (50-70%) of important herbal formulation, picroliv. Picroliv is reported to have hepatocurative effect against alfatoxin B1 induced hepatotoxicity in rats (Rastogi *et al.*, 2000). It also has immunostimulant activity against *Leishmania donovani* infection in hamsters (Puri *et al.*, 1992). P-I and P-II affect the activity of xenobiotic transport proteins implicated in multidrug resistance in neoplastic tissues (Najar *et al.*, 2009).

Picrosides are monoterpenes with an iridane skeleton. Monoterpenes are derived from GPP. GPP is synthesized 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). Cyclization of GPP yields iridoid; addition of glucose and cinnamate to iridoid yields P-I, whereas addition of glucose and vanillate to this moiety yields P-II.

Supply of GPP is critical in realizing the yield of terpenoids (Nogués *et al.*, 2006), therefore studies on regulation of gene expression in GPP biosynthesis is of immense significance. To understand molecular basis of picrosides biosynthesis, cloning of genes of the pathway and a study on their regulation of expression is central. Accordingly, the present work was undertaken with the following objectives:

I. Cloning and characterization of various genes involved in picrosides biosynthetic pathway

II. Spatio-temporal regulation of above genes of the pathway
The important findings of the present work are summarized below:

A. General aspects of picrosides accumulation/biosynthesis

A.1 Picrosides were reported to be present in the roots and rhizome of *P. kurrooa* (Verma, *et al.*, 2009; Ansari *et al.*, 1988). However, its presence in leaves was not established. Presence of picrosides in the leaf tissue will have implications in developing appropriate package and practice for harvesting the aerial parts and not uproot the whole plant so as to conserve this endangered species.

The analysis was performed on the plants maintained in green house as well as on those collected from the natural population growing at Rohtang Pass (District Kullu, Himachal Pradesh, India). Leaves (average of first to sixth leaf; apical leaf was designated as first leaf), rhizome and root had 1.52 mg, 1.11 mg and 0.07 mg of P-I, respectively per 100 mg of the tissue fresh weight. The values for P-II were 0.14 mg, 1.94 mg and 0.31 mg in 100 mg fresh weight of each of leaves, rhizome and root tissues, respectively.

A.2 The relative distribution of biomass in different tissues was studied to assess the overall distribution of picrosides. Fresh weight of leaves, rhizome and root of mature plant was 28.48 g, 13.91 g and 7.78 g, respectively. Corresponding dry weight for these tissues were 3.73 g, 3.13 g and 1.33 g, respectively in the same order.

A.3 Picrosides content was estimated in first to sixth leaf (apical leaf was designated as first leaf). First leaf had maximum and sixth leaf had minimum picrosides content.

A.4 Picrosides content was found to be higher in leaves, rhizome and root during light period (at 9:00 h and 13:00 h) as compared to the low light/dark period (at 5:00 h and 17:00 h).

A.5 The P-I content in leaves, rhizome and root was 2.32 mg, 1.22 mg and 0.10 mg (per 100 mg of fresh weight), respectively in the plants growing in its niche at Rohtang Pass.

A.6 All the above data unequivocally established the presence of picrosides in leaf tissue of *P. kurrooa* (Singh *et al.*, 2011).

A.7 Relative contribution of MVA and MEP pathway towards picrosides biosynthesis was not known in *P. kurrooa*. Fosmidomycin (50 μM, 100 μM and 200 μM), a specific inhibitor of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (dxr) of MEP pathway, and mevinolin (50 μM, 100 μM and 200 μM), a specific inhibitor
of 3-hydroxy-3-methylglutaryl-CoA reductase (hmgr) of MVA pathway was used separately to decipher the role of these pathways in picroside biosynthesis. Fosmidomycin (200 μM) reduced P-I content by 96.9% at 72 h of the treatment, whereas the content was increased by 1000% in the presence of mevinolin (200 μM). P-II content was decreased by 48% in the presence of fosmidomycin (200 μM), whereas an increase by 604.2% was recorded by the mevinolin (200 μM) treatment. These results implied a prominent role of MEP pathway in picroside biosynthesis, though a role of MVA pathway cannot be completely ruled out. Also, a cross-talk between the two pathways was evident.

A.8 Feeding of leaf tissue with [1-13C] glucose also showed the predominant role of MEP pathway in the biosynthesis of iridoid moiety. Incorporation of labeled carbon from [1-13C] glucose to iridoid moiety was found at C-6 and C-10 positions. Labeling at C-6 position suggested that MEP route was preferably used by *P. kurrooa* for biosynthesis of the GDP.

A.9 A role of ABA, MeJA, PEG, H2O2, SA and GA3 was examined in relation to picrosides content. ABA (200 μM) increased P-I but decreased P-II. MeJA (200 μM) did not alter the content of either P-I or P-II. PEG (15%) and H2O2 (20 mM) increased the content of both P-I and P-II. GA3 (200 μM) treatment did not alter the picrosides content, whereas SA (200 μM) decreased the content of both P-I and P-II.

B. Cloning of genes associated with picrosides biosynthesis pathway

B.1 DD was performed using the RNA isolated from control and PEG treated leaf tissue to clone various genes of the pathway. PEG modulated the picrosides contents and hence was expected to modulate the expression of genes involved in picrosides biosynthesis. DD yielded 43 cDNA fragment, and partial amplicons of *Pkthi, Phmgrp, Pkgdps* and *Pkdr*, along other novel genes and genes of unknown functions. DD, however, was expensive and time consuming technique and hence degenerate primer based approach was followed to clone rest of the genes associated with picrosides biosynthesis pathway.

B.2 Degenerate primers of various genes associated with MVA and MEP pathways were designed using sequence data of the corresponding cDNAs available at GenBank. By utilizing these primers, eight partial cDNAs were cloned as follow:
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*Pdks* (400 bp), *Pkmk* (410 bp), *Pkhdr* (450 bp), *Pkaclh* (400 bp), *Pkhmgs* (400 bp), *Pkipi* (400 bp), *Pkpa1* (230 bp) and *Pkcbl* (250 bp).

B.3 The partial sequences allowed designing of primers for Rapid Amplification of cDNA Ends (RACE) to clone full-length cDNAs. Nine full-length cDNAs were cloned as follow: *Pdks* (2317 bp), *Pdxr* (1767 bp), *Pkmk* (1674 bp), *Pkhdr* (1701 bp), *Pkaclh* (1545 bp), *Pkhmgs* (2241 bp), *Pkipi* (987 bp), *Pkgdps* (1434 bp) and *Pkthi* (1545 bp). The important characteristics of these genes are summarized in the following paragraph:

I. *Pdks* (GenBank accession number EU561005.1) was 2317 bp, consisting of a coding region of 2064 bp, 5' untranslated region (UTR) of 50 bp, 3' UTR of 203 bp including a poly A' tail of 23 bp. The polyadenylation signal AAGAAA was present at position 2155 bp. It encoded a putative Pdks of 687 amino acids (aa) with predicted molecular mass of 73.62 kilodalton (kDa) and isoelectric point (pI) of 8.60. Pdks possessed putative thiamine binding site. Characteristic signature motifs GDG(7-8)EX(3-4)AX(11-13)N and VGAL were present at position 185-216 aa and 234-238 aa, respectively.

II. *Pdxr* (GenBank accession number DQ347963) was 1767 bp consisting of a coding region of 1458 bp, 5' UTR of 115 bp, 3' UTR of 194 bp including a poly A' tail of 28 bp. The polyadenylation signal ATTAAA was present at position 1706 bp. It encoded a putative Pdxr of 486 aa with predicted molecular mass of 51.42 kDa and pI of 5.89. Pdxr possessed conserved Cys-Ser-X motif and proline rich region near the N-terminal (at 58–66 aa), which is characteristic of a functional PdXR.

III. *Kcmk* (GenBank accession number EF 199769) was 1674 bp, consisting of a coding region of 1395 bp, 5' UTR of 174 bp, and 3' UTR of 279 bp including a poly A' tail of 61 bp. The polyadenylation signal AATAAA was present at position 1436 bp. It encoded a putative Pkmk of 465 aa with predicted molecular mass of 44.46 kDa and pI of 5.87. Pkmk possessed characteristic CDP-ME binding motif K-L-N-L-F-L at position 164-168 aa and ATP binding motifs G-L-G-G-G and C-T-GR-G at positions 191-198 aa and 242-247 aa, respectively.

IV. *Pkhdr* (GenBank accession number EF199770.1) was 1701 bp, consisting of a coding region of 1266 bp, 5' UTR of 47 bp, 3' UTR of 277 bp
including a poly A⁺ tail of 27 bp. The polyadenylation signal CATAAA was present at position 1466 bp. It encoded a putative Pkhdr of 422 aa with predicted molecular mass of 51.90 kDa and pI of 5.96. Pkhdr possessed four conserved cysteine residues which play a vital role in catalytic activity.

V. *Pkaclh* (GenBank accession number DQ347964) was 1545 bp consisting of a coding region of 1245 bp, 5' UTR of 84 bp, 3' UTR of 246 bp including a poly A⁺ tail of 24 bp. The polyadenylation signal AATAAA was present at position 1436 bp. It encoded a putative Pkaclh of 405 aa, and predicted molecular mass of 41.41 kDa and pI of 6.33. Pkaclh possessed thiolase signature with consensus N-x(2)-G(2)-x[LIVM])-SA-x-G-H-P-x-[GAS]-x-[ST]-G near the C-terminus at position 350-366 aa. Thiolase active site was represented by motif GVGGVCNGgGgAsA at position 385-398 aa. These motifs have a role in catalytic activity of Pkaclh.

VI. *Pkhmgr* (GenBank accession number DQ 347962) was 2241 bp consisting of a coding region of 1683 bp, 5' UTR of 103 bp, 3' UTR of 452 bp including a poly A⁺ tail of 27 bp. The polyadenylation signal AGGAAAA was present at position 2198 bp. It encoded a putative Pkhmgr of 561 aa with predicted molecular mass of 60.17 kDa and pI of 6.82. Pkhmgr possessed HMG-CoA binding motif of an E83 loop, represented by consensus sequence DAMGXN at position 374-380 aa, and NAD(P) binding motif by GX2G2XT at position 526-534 aa. An EX3GX4P motif, reported essential for the HMG-CoA binding site, was present at position 251-261 aa.

VII. *Pkgdps* (GenBank accession number AY 866498) was 1434 bp consisting of a coding region of 1128 bp, 5' UTR of 113 bp, 3' UTR of 205 bp including a poly A⁺ tail of 33 bp. The polyadenylation signal TATAAA was present at position 1267 bp. It encoded a putative Pkgdps consisting of 375 aa with predicted molecular mass of 40.39 kDa and pI of 5.38. Pkgdps possessed conserved aspartate residue motifs DD(X)2-4,D present at 150-157 aa, the other two such motifs (DXXD) were present at 287-291 aa and 306-310 aa.

VIII. *Pkippi* (GenBank accession number EF 421829) was 987 bp consisting of a coding region of 657 bp, 5' UTR of 138 bp, 3' UTR of 192 bp including a poly A⁺ tail of 27 bp. The polyadenylation signal ATAAA was present at

IX.  

PKThi (GenBank accession number AY866497) was 1361 bp consisting of a coding region of 1065 bp, 5′ UTR of 70 bp, 3′ UTR of 226 bp including a poly A+ tail of 27 bp. The polyadenylation signal AAATAA was present at position 1198 bp. It encoded a putative PKThi of 355 aa with predicted molecular mass of 37.48 kDa and pI of 5.03. PKThi possessed GXGXXGLXXA motif with conserved glycine residue at aa position 94-103.

X.  

PKcaff could not be cloned to full length, despite several attempts. The 800 bp long sequence was assembled by aligning 5′ (500 bp) and 3′-RACE (1300 bp) sequences. It encompassed an open reading frame (ORF) of 700 bp. The start codon ATG could not be located suggesting that 5′ end was incomplete; however stop codon TAA was present at position 597 bp. The polyadenylation signal AATAAA was present at position 755 bp along with a poly A+ tail of 21 bp. Characteristic methyl transferase domain was present at position 1-190 aa.

XI.  

PKpal could not be cloned to full length, despite several attempts. The 862 bp long sequence was assembled by aligning 5′ (350 bp) and 3′-RACE (683 bp) sequence. It encompassed an ORF of 647 bp. The start codon ATG could not be located suggesting that 5′ end was incomplete; however stop codon TAA was present at position 647 bp. The polyadenylation signal AATTA was present at position 779 bp along with a poly A+ tail of 25 bp. Characteristic domain of phenylalanine ammonia lyase family was present at position 1-200 aa.

XII.  

PKhmgs could not be cloned to full length, despite several attempts. It could not be extended further by RACE. The 400 bp long sequence of pkhmgs was partial fragment. The start and stop codon could not be located in this sequence. It encoded a 128 aa of a putative pkhmgs.

C.  

Gene expression analysis

C.1  

In youngest leaves (first leaf), genes of the pathway exhibited lower expression during early hours of the day (5:00 hrs and 9:00 h) as compared to late hours (13:00 h and 17:00 h).
C.2 In the older leaves (fourth leaf), except for *Pkpal* and *Pkcaff* other genes did not exhibit any alternation in gene expression during early versus late hours.

C.3 Expression of genes in rhizome was similar at all time points of the day.

C.4 GA$_3$ up-regulated the expression of, *Pkhmgr, Pkpal* and *Pkcaff* at 24 h, whereas down-regulation was recorded at 48 h of the treatment as compared to the respective control. The expression of *Pkdxx, Pkdxr* and *Pkhdr* exhibited down-regulation at both the time intervals. Amplicons of *Pkcmk* and *Pkippi* did not exhibit any alternation in gene expression during the entire period of experimentation.

C.5 ABA down-regulated the expression of *Pkdxx, Pkhmgr, Pkdxx, Pkcaff* and *Pkgdps* at 48 h of the treatment. *Pkippi* exhibited up-regulation, whereas the expression of *Pkpal* was not altered.

C.6 MeJA up-regulated the expression of *Pkhmgr, Pkippi* and *Pkpals* at 24 h and 48 h of treatment as compared to the respective control. *Pkdxx* exhibited up-regulation at 24 h and down-regulation at 48 h of treatment. The down-regulation was exhibited by (i) *Pkhdr* at 24 h and 48 h, (ii) *Pkaech, Pkdxxr* at 48 h, and (iii) *Pkpgdp* at 24 h of the treatment.

C.7 H$_2$O$_2$ up-regulated the expression of *Pkdxx, Pkhdr* and *Pkcaff*, whereas *Pkgdps* and *Pkdxxr* exhibited down-regulation at all the time intervals as compared to the respective control.

C.8 PEG up-regulated expression of *Pkaech, Pkhmgr, Pkippi, Pkdxxr, Pkcmk* and *Pkcaff* and down-regulated the expression of *Pkdxx* and *Pkgdps*. Expression of *Pkhdr* and *Pkpals* was unaffected during the experimentation period.

C.9 SA up-regulated the expression of *Pkhmgr, Pkcmk, Pkdxxr, Pkippi, Pkpals* and *Pkcaff* whereas down-regulated the expression of *Pkaech, Pkdxxs* and *Pkgdps* as compared to respective control during the entire period of study.

**Conclusions**

The present work is the first endeavor to understand the molecular basis of picrosides biosynthesis in *P. kurrooa*. Presence of picrosides in leaf tissues suggested that aerial part of the plant can be used as a source of picrosides. Experiments utilizing pathway specific inhibitors and [1-13C] glucose showed the predominant role of MEP pathway in the biosynthesis of iridoid moiety of picrosides. The work for the first time reported nine full length cDNAs and three additional partial cDNAs associated with
picrosides biosynthetic pathway. The picrosides biosynthesis pathway was found to be modulated by various cues as evidenced by gene expression and picrosides content data. Data suggested a need to understand the regulation of various genes associated with condensation of cinnamoyl/vanilloyl moiety to iridoid/iridoid+glucose moiety. Also, it will be worthwhile to identify glucosyltransferase(s) involved in transfer of glucose to iridoid moiety. Though, the supply of GDP was shown to be critical in realizing the yield of terpenoids (Mahamud and Croteau, 2002), an interesting indication in the present work was that GDP biosynthesis may not always be a factor deciding the yield of monoterpane picrosides. It appears that the following reactions might also play a vital role in determining picrosides content:

(i) reactions associated with cyclization of GDP,
(ii) glucosyltransferase(s) involved in transfer of glucose to the iridoid moiety,
(iii) reactions determining availability of cinnamoyl/vanilloyl, and
(iv) reactions responsible for condensation of cinnamoyl/vanilloyl moiety to iridoid/iridoid +glucose.