CHAPTER 5

DISCUSSION

*Picrorhiza kurroa* is an important medicinal herb but inspite of broad range of medicinal values of its major iridoid glycosides Picroside-I and Picroside-II, the biology of picrosides biosynthesis has been partially understood till date. Rational approaches towards enhancing the production of picrosides has been greatly impeded by our poor understanding of the regulatory and metabolic pathways as well as missing links underlying the biosynthesis of these compounds. Therefore, this research work was carried with an aim of elucidating biosynthetic pathways and achieve thorough understanding of molecular basis of picrosides biosynthesis and natural variation for major compounds in *P. kurroa*. This research work has provided initial leads which can be taken forward to carry out any genetic improvement strategy for enhancing picrosides content in *P. kurroa*.

First, we carried out the quality assessment of *P. kurroa* plant material being sold in major herbal drug markets of North India (Delhi, Amritsar and Manali) by estimating major chemical constituents, Picroside-I and Picroside-II in rhizomes which are used in the preparation of herbal drugs. The quality assessment of raw material is important so that appropriate plant material should be used in the preparation of *P. kurroa* formulations with desired efficacy. Further, variation in picrosides contents of twenty six *P. kurroa* accessions from different geographical locations of North-Western Himalayas was analysed. It was observed that there is a wide range of variation in picrosides content in *P. kurroa* accessions.

Next, the complete biosynthetic pathway including all genes catalysing the corresponding enzymatic steps was deciphered using information from NGS transcriptomes of *P. kurroa* which proved to be of paramount importance to get a clear picture of the dynamics of Picroside-I and Picroside-II biosynthesis. Transcriptome analysis has been helpful in accelerating gene discovery, expression analysis, improving genome annotation, identifying splice variants, and identification of molecular markers such as SNPs, SSRs, etc. in different plant species [139]. Whole transcriptome analysis is of growing importance in understanding how altered expression of genetic variants contributes to different metabolic pathways. Greater insights into biological pathways and molecular mechanisms that regulate cell fate, development and secondary metabolites synthesis can be gained through genome-wide RNA expression analysis. Further, key genes involved in picrosides
biosynthesis were shortlisted through expression analysis in different tissues and genotypes of *P. kurroa*. As picrosides are biosynthesized through a combined biosynthetic route involving MEP, MVA, iridoid and phenylpropanoid pathways, the contribution of these pathway modules was ascertained using inhibitor studies. The role of MEP pathway as the major contributor of geranyl pyrophosphate (GPP), the iridoid backbone for picrosides biosynthesis in *P. kurroa* has been ascertained. After GPP biosynthesis, iridoid and phenylpropanoid pathways play equally important role in picrosides biosynthesis by providing catalpol and cinnamate/vanillate moieties, respectively. The complete biosynthetic pathway of Picroside-I and Picroside-II including corresponding enzymatic steps has been deciphered for the first time in *P. kurroa*. Finally, molecular characterization of *P. kurroa* accessions varying for Picroside-I and Picroside-II contents was done using SSR markers to assess variation at genetic level. The significant outcomes have been discussed below.

5.1 Quality assessment of *P. kurroa* plant material from herbal drug markets

There has been an increasing public interest and acceptance of herbal medicines in both developing and developed countries. In developing countries, about 80% of the population uses herbal medicines as their primary source of healthcare [140]. But several factors affect the quality of herbal drugs such as active principles in most cases are unknown either qualitatively or quantitatively or both, plant materials are chemically variable, existence of chemo-cultivars and chemo-varieties, variable source and quality of raw material, etc. [141]. The methods of harvesting, drying, storage, transportation, and processing also affect herbal quality. There are no official standards available at present for herbal raw material and preparations. Herbal raw materials and remedies derived from them contribute substantially to the global market, therefore assessment of its quality is necessary.

Quality assessment of *P kurroa* plant material was done from herbal drug markets of North India which contributes to the major supply of *P. kurroa* raw material with Delhi market alone contributing to more than 10,000 kg annually [142]. Market samples varied in quality with respect to major chemical compounds, Picroside-I and Picroside-II. The sample from Himachal Pradesh showed highest picrosides content, whereas inspite of the good physical appearance, the plant material from China showed least picrosides content. The higher picrosides contents in rhizomes of Himachal Pradesh might be due to the reason that factors affecting picrosides biosynthesis and accumulation such as altitude, temperature, soil
composition, different biotic and abiotic stresses, etc. are more favourable in Himachal Pradesh in comparison to China. This suggests that physical appearance should not be the only criteria for judging the quality of plant material, rather it should be judged on the basis of major phytochemical constituents of the plant. *P. kurroa* plant material is added in herbal formulations in different amounts (on weight basis) and not on the basis of content of marker compounds. Proper concentration of Picroside-I and Picroside-II is required for the preparation of herbal drug formulations from *P. kurroa*, therefore, it is necessary to analyse the quality of plant material so that herbal drug formulations with desired pharmacological efficacy can be produced. Quality assessment of market samples has been done for the first time for *P. kurroa* plant material. No reports on quality assessment of raw material for marker compounds/major chemical constituents exist even in any other medicinal plant species. This would bring the focus of researchers working on other important medicinal plants to determine the quality of respective plant materials being sold in herbal drug markets.

5.2 Sites of biosynthesis and accumulation of picrosides

The initial requirement for deciphering and understanding the complete biosynthetic machinery of picrosides biosynthesis in *P. kurroa* was to identify the sites of biosynthesis and accumulation of Picroside-I and Picroside-II. Estimation of Picroside-I and Picroside-II contents vis-à-vis morphogenic and developmental growth of shoots, rhizomes and roots has provided insights about the sites of biosynthesis and storage of picrosides in *P. kurroa* [22, 23, 33]. Understanding differential biosynthesis and accumulation of picrosides would also assist in regulating the quality of plant material for herbal drug formulations. Biosynthesis of Picroside-I takes place preferentially in shoots whereas Picroside-II in roots or stolons. Shoots of tissue cultured *P. kurroa* plants as well as field grown plants were found to contain only Picroside-I whereas Picroside-II was detected only in roots of field grown plants. Rhizome accumulates both Picroside-I and Picroside-II which increases with rhizome age as it is a storage organ. Moreover, different sections of the same rhizome are reported to contain variable amount of picrosides contents with highest content in the uppermost section followed by middle and lower sections [23]. Through all these studies it was suggested that the primary site of biosynthesis of Picroside-I is in green leaf cells of young shoot buds, whereas the primary site of biosynthesis for Picroside-II is either in the young rhizome (stolons) or root cells because it was detected in both the tissues [6, 22].
Differential biosynthesis and accumulation of secondary metabolites in different tissues has also been reported in other plant species. For example, the accumulation of hypericin and hyperforin occurs in leaves and inflorescence of *Hypericum perforatum*, respectively [143]. The accumulation of withanolide occurs in shoot tips and leaves of *Withania somnifera* [144]. Tuberous roots of *Aconitum heterophyllum* are reported to produce and accumulate atisine whereas it is absent in shoots [145]. Differential biosynthesis and accumulation of major phytochemicals swertiamarin, amarogentin and mangiferin has been observed in roots leaves and flowers of *Swertia chirayita* [146]. Recently, differential accumulation of rutin, betulin and betulinic acid in roots, stems, leaves and fruits of *Morus alba* has been reported with highest content of rutin and betulinic acid in leaves and betulin in roots [147]. From all these studies, it is inferred that biosynthesis and accumulation of secondary metabolites is tissue and organ specific. Therefore, morphological and biochemical phenotypes are of great use for studying the genetic regulation of the formation of metabolites and their functions in plants [148].

5.3 Picrosides content variation in *P. kurroa* accessions

*P. kurroa* accessions from different geographical locations in Himachal Pradesh were quantified for variation in Picroside-I and Picroside-II contents. Shoot as well as rhizome tissues were quantified. Our study showed that shoot tissue contained consistently same amount of Picroside-I content in field grown plants as well as greenhouse plants at different time periods whereas rhizome tissue showed variable picrosides content. The accumulation pattern of Picroside-I content remained consistent in shoot tissues of the same accession in successive repetitions during different years (2011-2013) whereas rhizomes showed inconsistent contents for the same accession which may be due to difference in age and developmental stage of rhizomes at the time of collection. Rhizomes being a storage organ keep on accumulating picrosides with age whereas there are no significant differences in Picroside-I content in shoots of different ages [23]. Consistency in Picroside-I content in shoot tissue has been observed in our previous study where shoots of different age groups (1-3 years) showed negligible variation in Picroside-I content but rhizomes of the same plants showed an increase of ~1.4 folds in both Picroside-I and Picroside-II contents from 1-3 years [23]. Significant increase in picrosides content in rhizomes occurred with increase in age. This highlights the importance of selection of appropriate tissue for assessing extent of genetic variation contributing to variation in picrosides contents, which would be helpful in the identification and selection of superior chemotypes. Moreover, selection of
appropriate tissue and uniformity in selection is essential for the preparation of herbal drug formulation of desired efficacy. Differences in environment are not the only source of variation for secondary metabolites. It has been shown that genetic effects, along with the interaction between environmental and genetic effects, are also significant causes of secondary metabolite variation [40]. The variation in picrosides biosynthesis and accumulation may be due to the environment or genotype or a combination of both. Therefore, to nullify the effect of environmental factors and ascertain that the variation in picrosides content is not just due to environmental factors but due to differences at the genetic level, the quantification of *P. kurroa* accessions growing in controlled environment in green house and natural environment in a nursery was done and the results showed that Picroside-I content remained similar across two environmental conditions i.e. greenhouse and field condition (Jagatsukh, 1900 meters asl, 2011-2013).

**5.4 Deciphering complete biosynthetic pathway of Picroside-I and Picroside-II in *P. kurroa***

The preliminary requirement for discerning biosynthetic machinery of picrosides was to achieve knowledge on complete picrosides biosynthetic pathway and its corresponding genes at each enzymatic step. Moreover, any metabolic engineering effort would require in depth understanding of the pathway and genes that contribute to the biosynthesis of picrosides. The biosynthetic pathway of picrosides was elucidated by Kumar et al. [28] but genes corresponding to enzymatic steps were not identified. Our study is an initial endeavour towards deciphering the complete biosynthetic pathway including corresponding enzymatic steps for picrosides biosynthesis in *P. kurroa*. Biosynthesis of picrosides occurs through an integrated biosynthetic route involving different modules, non-mevalonate (MEP), mevalonate (MVA), phenylpropanoid and iridoid pathways. Picrosides are classified as Picroside-I and Picroside-II based on functional group moieties; Picroside-I having cinnamate moiety and Picroside-II having vanillate moiety [24] both derived from phenylpropanoid pathway. Iridoid backbone is derived from geranyl pyrophosphate (GPP) which is synthesized by head to tail condensation of isopentenyl pyrophosphate (IPP) and its allelic isomeric dimethylallyl diphosphate (DMAPP) via cytosolic mevalonate (MVA) and/or plastidic (MEP) pathway [25, 26]. There are 15 enzymatic steps from MEP and MVA pathways involved in the formation of GPP, the precursor for iridoid backbone biosynthesis. Out of 15, only sequences of 10 genes were available in GenBank for *P. kurroa*. Remaining 5 genes, ISPD, MECPS, HDS, HMGS and PMK were cloned in *P. kurroa* by Pandit et al.
and expression analysis of all 15 genes was done vis-à-vis Picroside-I and Picroside-II contents in different tissues of *P. kurroa*. Multiple genes, HMGS, HMGR, MVK, PMK, MVDD, GDPS, DXPS, ISPD, MECPS and ISPH had shown elevated expression levels in relation to picrosides contents [33]. Eight genes of MEP and MVA pathways, DXPS, DXPR, ISPE, ISP, ACTH, HMGR, IPPI and GDPS were cloned to full length followed by expression analysis in different tissues of *P. kurroa* [24]. Expression analysis of MEP and MVA pathway genes has been studied in other medicinal plant species in relation to secondary metabolites contents, for example artemisinin in *Artemisia annua* [107], shikonins in *Arnebia euchroma* [36], atisine in *Aconitum heterophyllum* [145], swertiamarin and amarogentin in *Swertia chirayita* [146], rutin, betulin and betulinic acid in *Morus alba* [147].

The genes of other modules of the picrosides biosynthetic pathway i.e. iridoid and phenylpropanoid pathway were not known in *P. kurroa*. The genes of phenylpropanoid pathway were mapped to the biosynthetic pathway using transcriptomic information as these were known in other plants species. The expression analysis of phenylpropanoid pathway genes for shikonins content in *Arnebia euchroma* was carried out by Singh et al. [36] and three genes, PAL, C4H and 4-coumaroyl-CoA ligase were shown to exhibit positive correlation with shikonins content. Partial sequences of two genes of phenylpropanoid pathway, PAL and COMT (CAM) were cloned in *P. kurroa* followed by expression analysis in different tissues. (Singh et al. 2012). Multiple genes of phenylpropanoid pathway has shown to be positively correlated with podophyllotoxin (PD, CM, CMT, C4H) in *Podophyllum hexandrum* [149], mangiferin (EPSPS, DHQS, PAL, ADT, CM, DAHPS, DHQD and CS) in *Swertia chirayita* [146] and flavonoids in *Morus alba* [147].

Further, the first seven enzymatic steps of iridoid pathway i.e. GS, G10H, 10HGO (10HD), IS, MC, CPM and UGT have also been recently identified during our study in medicinal plant species such as *Catharanthus roseus, Gardenia jasmonides* and *P. kurroa* [137, 138, 34] while genes encoding rest of the steps were unknown. Therefore, the identification of enzymatic steps of iridoid branch of the pathway, not known earlier was done, however functional characterization is required to support their proposed functions in *P. kurroa*. The higher expression of gene transcripts for corresponding enzymes vis-à-vis Picroside-I and Picroside-II contents and their involvement in secondary metabolism in other plant species suggested their possible role in catalysing the required enzymatic reactions in picrosides biosynthesis pathway. Geraniol synthase (GS) is the first and the committed step which
initiates the iridoid branch of pathway by formation of geraniol from GPP. GS has been cloned to full length and functionally characterized in *Catharanthus roseus* [150] by overexpression in *E. coli* as well as heterologous expression in *S. cerevisiae*. Geraniol 10-hydroxylase (G10H) is involved in the biosynthesis of iridoid monoterpenoids and several classes of monoterpenoid alkaloids are found in diverse plant species. It is reported to play a regulatory role in monoterpene indole alkaloids biosynthesis in *Catharanthus roseus* which was proved by enzyme assay and heterologous expression in yeast [151]. G10H from *Swertia mussottii* was cloned to full length, heterologously expressed in *E. coli* and *Pichia pastoris* and in vitro enzyme activity of was analysed. Further, overexpression of G10H in *S. mussottii* resulted in an increase in swertiamarin content [152]. 10HGO is one of the key enzymes involved in monoterpenoid indole alkaloids (MIAs) in *Catharanthus roseus* and its role was validated through cloning and functional characterization [153]. Iridoid synthase (IS) was identified in *Catharanthus roseus* and its role in iridoid biosynthesis was supported by biochemical assays, gene silencing, co-expression analysis and localization studies [154]. Monoterpene cyclase (MC) has been reported in iridoids biosynthesis in *Gardenia jasmonides* [138]. Molecular characterization of UGTs has been done in *P. kurroa* and functional characterization in *Gardenia jasmonides*. Two UGTs from *P. kurroa* were cloned to full length and differential expression pattern vis-à-vis picrosides content demonstrated the role of UGTs in picrosides biosynthesis [176]. A UGT was cloned in *Gardenia jasmonides* and identified to be involved in iridoid biosynthesis in *Gardenia* fruits which was supported using enzyme assay and heterologous expression [138].

The eighth identified enzymatic step of the iridoid pathway is aldehyde dehydrogenase (ALD). Aldehyde dehydrogenase is an enzyme that catalyzes the oxidization of aldehydes to carboxylic acids [155]. A reaction similar to this enzymatic step (conversion of boschnaloside to 8-epideoxyloganic acid) has been shown to be catalyzed by ALD in the conversion of dihydroartemisinic aldehyde to dihydroartemisinic acid in *Artemisia annua* [107]. Also, expression level of ALD gene transcript was shown to be in accordance with artemisinin content in *Artemisia annua* and other species of *Artemisia* [156]. ALDs have been biochemically characterized from different plant species such as maize, pea and *Arabidiopsis thaliana* [157, 158]. The next enzymatic reaction (conversion of 8-epideoxyloganic acid to mussaenosidic acid) is catalyzed by flavanone 3-dioxygenase/hydroxylase (F3D) which catalyzes a similar reaction in conversion of naringenin to dihydrokaempferol in *Petunia* [159]. F3D has been cloned and functionally
characterized from *Ginkgo biloba* and *Petunia* [160, 159]. F3D is reported to be involved in flavanoids biosynthesis in many plant species like *Ginkgo biloba*, *Camellia sinensis*, *Juglans nigra*, etc. and transcript abundance of F3D has been correlated with the accumulation of flavonoids and flavanols content in different plant tissues [160, 161, 162].

The next enzymatic step of the biosynthetic pathway has been identified as 2-hydroxyisoflavanone dehydratase (2HFD) which involves the removal of H₂O molecule in the conversion of mussaenosidic acid to deoxygeniposidic acid. 2HFD catalyzes a similar reaction in the conversion of 2-hydroxyisoflavanone to isoflavone in *Pueraria lobata* [163]. 2HFD is involved in isoflavone biosynthesis in legumes like *Glycine max* [164] and in cell cultures of *Pueraria lobata* [163] and it has also been identified as a critical determinant of isoflavone productivity in hairy roots of *Lotus japonica* [165]. Biochemical characterization of 2HFD in above studies have also validated the catalytic activity of this enzyme in plants.

Next step is conversion of deoxygeniposidic acid to geniposidic acid which involves addition of OH group. A similar hydroxylation reaction occurs in the conversion of deacetoxycephalosporin C to deacetylcephalosporin C which is catalyzed by deacetoxycephalosporin-C hydroxylase (DCH). DCH is involved in cephalosporin C biosynthesis [166, 167]. This step is predicted to be catalyzed by an enzyme like DCH. The next step is conversion of geniposidic acid to bartsiosate which involves removal of CO₂ group catalyzed by a decarboxylase enzyme. Two decarboxylases, uroporphyrinogen decarboxylase (UPD) and UDP-glucuronic acid decarboxylase (UGD) involved in secondary metabolites biosynthesis were identified in the transcriptomes of *P. kurroa* which can catalyse such type of reaction. UGD is reported to catalyze the conversion of UDP-D-glucuronate to UDP-D-xylene in barley [168] and rice [169]. Uroporphyrinogen decarboxylase is associated with the activity of enzymes involved in tetrapyrrole biosynthesis and pathogen defense response in *Nicotiana tabacum* [170, 171]. Next step is the conversion of aucubin to catalpol which involves oxidation reaction to form epoxide.

This type of epoxidation reaction is catalyzed by squalene epoxidase/monooxygenase (SQE/SQM) in oxygenation step of phytosterol and triterpenoid saponin biosynthesis in *Panax ginseng* and suggested to be the rate limiting step of triterpene biosynthetic pathway [172]. Molecular cloning and characterization of SQE has been done in *Panax notoginseng* and differential expression pattern of this enzyme was observed in different tissues with highest expression in roots [173]. It is also involved in ginsenoside biosynthesis in *Panax ginseng* [172, 174], triterpenoids in *Uncaria tomentosa* [175] and withanolides in *Withania somnifera* [176]. The last and the most important step in picrosides biosynthetic pathway is
the conversion of catalpol to Picroside-I and Picroside-II via transfer of cinnamate and vanillate moieties, respectively which takes place through acyl group transfer. This acylation reaction can be catalyzed by acyltransferase (ACT) enzyme. An acyltransferase known as anthocyanin acyltransferase was identified from the transcriptome which is reported to catalyze regiospecific acyl transfer from acyl-CoA to the sugar moiety of anthocyanins [177]. The role of ACTs to catalyze acyl group transfer in plants have been validated through functional characterization and heterologous expression [177]. ACTs are reported to be involved in anthocyanin biosynthesis in *Gentiana triflora* [178]. This stepwise identification of enzymatic steps through literature survey resulted in the elucidation of complete biosynthetic pathway of Picroside-I and Picroside-II biosynthesis in *P. kurroa*.

The identified enzymes belong to cytochrome P450 family which possess broad substrate specificity i.e. same enzyme can bind to different substrates but enzyme kinetics vary considerably from one substrate to another [179, 180]. Therefore, these enzymes inspite of preferentially using other substrates can also catalyse the similar reactions for picrosides biosynthesis using pathway intermediates as their substrates. The genes were mapped to all the enzymatic steps but it was observed that multiple paralogs/copies of pathway genes were present in *P. kurroa* transcriptomes. Paralogs are the homologous sequences which are highly similar but may be different functionally. The previous studies done in *P. kurroa* have not considered the importance of selection of appropriate paralog for expression analysis of picrosides biosynthetic pathway genes [24, 27, 34, 116]. But, each paralog of the same gene can have different level of expression in the same plant tissue. Therefore, identification of functionally correct paralog is a prerequisite for accurate quantification of transcript abundance of genes involved in picrosides biosynthesis. Therefore, appropriate paralogs of each pathway gene were shortlisted before proceeding for further expression analysis in different tissues of *P. kurroa*.

**5.5 Multiple genes correlate with picrosides content**

The expression analysis was done on *P. kurroa* shoot and root tissues varying for Picroside-I and Picroside-II contents. Analysis of differential RNA expression provides greater insights into biological pathways and molecular mechanisms that regulate cell fate, development and secondary metabolites synthesis. Reverse transcriptase-quantitative PCR (RT-qPCR) analysis revealed that most of the genes of picrosides biosynthetic pathway had
relatively higher expression in field grown tissues of *P. kurroa* containing Picroside-I or Picroside-II compared to tissue culture grown plants having negligible or no picrosides content at all. Genes, HMGS, HMGR, MVK, PMK, MVDD, GDPS, DXPS, ISPD, ISPE, MECPS, ISPH from MEP and MVA pathways [33], GS, G10H, CPM, ALD, F3D, 2HFD, DCH, UPD/UGD, SQM, ACT from iridoid pathway and DQS, DQD, QSD, SK, EPSPS, CM, APD, TAT, PAL from shikimic acid/phenylpropanoid pathway showed significantly higher folds expression vis-à-vis picrosides content. Various studies have reported expression of multiple genes of MEP, MVA, phenylpropanoid and iridoid pathways to be positively correlated with the terpenoid biosynthesis; shikonins in *Arnebia euchroma* [36], artemisinin in *Artemisia annua* [107], MIAs in *Catharanthus roseus* [181], flavonoids biosynthesis in *Fagopyrum* species [182], lignin biosynthesis in *Arabidopsis thaliana* [183], taxol biosynthesis in *Taxus media* [184], atisine in *Aconitum heterophyllum* [145], podophyllotoxin in *Podophyllum hexandrum* [149] and swertiamarin, mangiferin and amarogentin biosynthesis in *Swertia chirayita* [146]. Geraniol synthase (GS) is an important enzyme which initiates monoterpenoid branch of the pathway in *Catharanthus roseus* [150] and the involvement of this enzyme in MIAs biosynthesis in *Catharanthus roseus* has been validated by molecular and *in planta* characterization where virus induced gene silencing and overexpression of GS resulted in decreased and increased accumulation of MIAs (catharanthine and vindoline), respectively [185]. G10H is reported to be a rate-limiting enzyme for biosynthesis of TIAs in *Ophiorrhiza pumila* [186] alongwith its importance in iridoid monoterpenoid swertiamarin biosynthesis in *Swertia mussotii* [152] and MIAs biosynthesis in *Catharanthus roseus* [187]. EPSPS, SK and PAL are also reported to be important regulatory enzymes of shikimate/phenylpropanoid pathway [188-190].

### 5.6 Key genes identified through expression analysis in high versus low Picroside-I content accessions (genotypes) of *P. kurroa*

In initial expression analysis, multiple genes showed higher expression for Picroside-I or Picroside-II or both in field grown plants w.r.t. tissue cultured plants. But, the expression analysis in tissues of different stages or different environmental conditions is not that much informative and do not provide a clear picture of the genes involved in the biosynthesis of a particular metabolite. In such cases, the variation in expression level of genes may be due to tissue type variation and biosynthesis of several other secondary metabolites, in addition to picrosides. The expression analysis of genes in the same tissue type of different plants varying for a particular metabolite grown in a similar environmental conditions would
uniquely represent the expression status of genes for that metabolite. For example, genes playing key role in atisine biosynthesis in Aconitum heterophyllum have been identified by analysing expression status of MEP and MVA pathway genes in roots of different genotypes having differential accumulation of atisine [145]. Similarly, key genes associated with artemisinin biosynthesis were identified by comparative expression analysis of artemisinin biosynthetic pathway genes in eight different species of Artemisia varying in artemisinin contents grown under similar environmental conditions [156]. Therefore, to ascertain whether the elevated levels of transcripts of pathway genes are only affecting the biosynthesis of picrosides uniquely, the expression status of genes was further studied between shoots of P. kurroa genotypes that were varying for Picroside-I. High and low Picroside-I content genotypes of P. kurroa (one each) grown for 3 years in the controlled environment in greenhouse were used for comparative expression analysis so as to reflect genetic differences contributing to the increase or decrease of gene transcripts rather than tissue type or developmental stage. It was observed that most of the genes which showed higher expression in field grown tissues w.r.t. their tissue cultured counterparts did not show significant difference in expression between high versus low content genotypes, thereby suggesting that all the genes which showed higher expression initially might be contributing to the biosynthesis of secondary metabolites other than the picrosides. The overall expression of genes decreased significantly after comparative analysis between shoots of high versus low Picroside-I content genotypes and relative transcript amounts of only seven genes remained higher in high content genotype. The additional analysis on high versus low content genotypes, shortlisted seven important genes, PMK, ISPE, 2HFD, DXPS, ISPD, EPSPS and SK thereby suggesting their major contribution in picrosides biosynthesis. Among these seven genes, four of MEP/MVA pathway ISPD, DXPS, ISPE and PMK are reported to be positively correlated with picrosides content in P. kurroa [33, 24] and DXPS is a well-known regulatory enzyme of MEP pathway [191, 192]. DXPS has been shown to be responsible for the monoterpenoid production and Muscat flavour in Vitis vinifera [193] and over expression of this gene upregulated monoterpane production in Nicotiana tabacum [193]. DXPS and ISPD are known to regulate alkaloids and secoiridoids biosynthesis in Gentiana macrophylla and C. roseus [194]. EPSPS is a key regulatory gene of shikimate pathway [188] which is associated with herbicide tolerance [195, 196] and biosynthesis of secondary metabolites [197, 198]. EPSPS was shown to have increased expression level in Swertia chirayita in relation to mangiferin content [146]. SK is an important regulatory gene in secondary metabolism as it has been suggested that plant SKs act as regulatory points for
the shikimate pathway [189]. It has been reported that 2-hydroxyisoflavanone dehydratase plays a key role in regulation of isoflavone biosynthesis as its overexpression resulted in accumulation of daidzein and genistein in *Lotus japonicas* [165]. Majority of these key genes belonged to MEP pathway. Also higher expression of two genes of shikimic acid/phenylpropanoid pathway and one gene of iridoid pathway, among seven key genes, highlights the importance of these modules of the biosynthesis of picrosides in *P. kurroa*. This suggests that each module of the pathway is important in contributing to the structures of parental compounds, Picroside-I and Picroside-II in *P. kurroa*.

These genes are thus suggested to be playing important role in controlling the biosynthesis of Picroside-I and Picroside-II in *P. kurroa*. It would be beneficial if multiple genes from different modules of the biosynthetic pathway can be targeted instead of a single gene because secondary metabolism gets regulated at multiple steps. It has been reported in various plant species that co-overexpression of multiple genes resulted in significant enhancement of secondary metabolites rather than single gene, for example co-overexpression of HMGR and/or GGPPS as well as DXS elevated tanshinone accumulation levels in *Salvia miltiorrhiza* [199], overexpression of cytochrome P450 monooxygenase alongwith CPR and HMGR with amorpha-4,11-diene synthase enhanced artemisinin content in *Artemisia annua* [200, 201], co-overexpression of seven key gene cassette enhanced ketocarotenoid accumulation in *Brassica napus* [202]. Co-overexpression of IPP isomerase and limonene synthase resulted in accumulation of increased number of secondary metabolites in *Mentha spicata* [203] and co-overexpression of G10H and strictosidine synthase in *Ophiorrhiza pumila* enhanced camptothecin [186]. Alternatively, common transcription factors regulating the expression of these multiple key genes can be identified and overexpressed to elevate their expression for enhancing the secondary metabolites production.

### 5.7 Inhibitor studies revealed MEP pathway as a major contributor for picrosides biosynthesis

Inhibitor studies play an important role in determining the contribution of a particular pathway step/module in the biosynthesis of secondary metabolites. The genes of major contributing pathways can be overexpressed for increased production of secondary metabolites and the pathways which are not contributing significantly can be blocked so that the flux can be diverted towards a major pathway. Since terpenoids are derived from GPP
that can be synthesized both from cytoplasmic MVA and plastidic MEP pathways [204], it is important to study the regulation of these two pathways as feeders of GPP.

Four inhibitors targeting important enzymatic steps of the MEP/MVA and phenylpropanoid pathways were selected to assess their effect on picrosides accumulation. The inhibitor concentrations were chosen based on previous reports [205-208, 35]. Mevinolin and fosmidomycin are highly specific inhibitors of MVA (HMGR) and MEP (DXPR) pathways, respectively [209, 210]. These two inhibitors were selected to rule out whether MVA and/or MEP pathway contributes in picrosides biosynthesis. Fosmidomycin produced drastic inhibition of up to 90.6% in Picroside-I accumulation whereas mevinolin resulted in slight (17%) inhibition. The higher inhibition in Picroside-I content by fosmidomycin in comparison to mevinolin suggested that the MEP pathway plays a major role in the production of GPP, the precursor for iridoid backbone biosynthesis (Figure 5.1). Picrosides are monoterpenoids and our results are in accordance with the previous findings that monoterpenoids have non-mevalonate (plastidial) origin and monoterpenoid synthases are localized to plastids [211]. It has also been reported by Eisenreich et al. [212] that MEP pathway is a predominant contributor for monoterpenoid biosynthesis, however, crosstalk occurs between two pathways [213]. Our previous reports have also suggested the predominant role of MEP pathway in picrosides biosynthesis as majority of the MEP pathway genes were highly expressed in relation to picrosides content [33]. Sood and Chauhan [22] have also highlighted the importance of plastids (chloroplasts) by showing that the biosynthesis of Picroside-I occurs only in in vitro cultured leaf and stem segments but not in undifferentiated callus cultures. Callus cultures lack chloroplasts and hence, Picroside-I can be attributed to the absence of proper cell organization and programming of cell machinery involved in the biosynthesis of Picroside-I.

The other inhibitors, glyphosate and AOA were selected targeting the shikimate/phenylpropanoid pathway enzymes. Glyphosate, a broad spectrum herbicide which competitively inhibits EPSPS [214] and AOA acts as an inhibitor of PAL [207] which is an important regulatory enzyme in phenylpropanoid pathway. Shikimic acid/phenylpropanoid pathway has a major contribution in picrosides biosynthesis, thereby, providing cinnamate and vanillate moieties for Picroside-I and Picroside-II, respectively. The decrease in Picroside-I biosynthesis by inhibiting shikimic acid/phenylpropanoid pathway enzymes confirms major contribution of this component of pathway in picrosides biosynthesis in *P. kurroa* (Figure 5.1). Glyphosate is reported to inhibit secondary
metabolites content in soyabean and buckwheat [206, 215]. AOA treatment resulted in decreased accumulation of phytoalexins in banana [208] and 2-hydroxy-4-methoxybenzaldehyde in *Hemidesmus indicus* roots [216].

**Figure 5.1** Representation of effect of different enzyme inhibitors on Picroside-I accumulation in *Picrorhiza kurroa*. Enzyme inhibitors point towards MEP route as a major contributor for picrosides biosynthesis.

The inhibition pattern was similar for inhibitors fosmidomycin, glyphosate and AOA where higher inhibition was observed at 30th day than 15th day of incubation for all concentrations. Mevinolin showed opposite inhibition pattern as higher inhibition was observed at 15th day than 30th day. This might be due to the fact that mevinolin is not a strong inhibitor in our case. Therefore, most of it is getting used up till 15th day, hence not showing significant inhibition at 30th day. On the basis of above observations fosmidomycin, glyphosate and AOA proved to be potent inhibitors whereas mevinolin as a weak inhibitor of picrosides biosynthesis in *P. kurroa*.

Inhibitor studies have also been reported in other plant species to determine the relative contribution of MEP and/or MVA pathway in the biosynthesis of respective secondary metabolites. Studies with mevinolin and fosmidomycin as inhibitors of MVA and MEP pathway, respectively suggested MVA as a preferred route of GPP supply for shikonins.
biosynthesis in *Arnebia euchroma* [36]. Mevinolin was found to be more effective in inhibiting shikonins production whereas fosmidomycin treatment could not inhibit shikonins production significantly, thereby suggesting the major contribution of mevalonate pathway in the biosynthesis of shikonins in *Arnebia euchroma*. Relative contribution of MVA versus MEP pathway towards ginsenosides biosynthesis in *Panax ginseng* has also been assessed by using mevinolin and fosmidomycin. It was demonstrated that both pathways are involved in ginsenoside biosynthesis, based on the analysis of the effects from suppressing either or both of the pathways on ginsenoside accumulation in *Panax ginseng* hairy roots. When MEP or MVA pathways were inhibited separately, no significant inhibition in ginsenosides accumulation was observed but inhibition of both pathways together resulted in decreased production of ginsenosides which suggested that both the pathways are equally involved in ginsenosides biosynthesis [217]. The role of MEP/MVA pathways in paclitaxel and baccatin III accumulation in suspension cultures of *Taxus baccata* has also been determined [35]. The presence of fosmidomycin inhibited the biosynthesis of paclitaxel as well as baccatin III to a greater degree than that of mevinolin which indicated that non-mevalonate pathway was the main source of terpenoid precursor, GGPP for the biosynthesis of both taxanes.

The inhibitor treatment thus resulted in decrease in picrosides biosynthesis. We further looked at whether the expression of biosynthetic pathway genes, which are targets of corresponding inhibitors, is also affected or not. The expression of upstream and downstream genes along with target genes was analysed. The inhibitors, fosmidomycin, mevinolin, glyphosate and AOA are competitive inhibitors which are highly specific for their target genes. Therefore, these inhibitors did not show significant decrease in expression of transcripts because these might be affecting the genes only at enzymatic level but are not producing any effect at the transcriptional level. It has been reported in *Arabidopsis* that inhibitor treatment did not show significant effect on the expression of genes involved in sterol, chlorophyll and carotenoid metabolism, thereby indicating that posttranscriptional processes might be playing an important role in regulating the flux through isoprenoid metabolic pathways [218].

**5.8 Distribution of SSRs in *P. kurroa* transcriptomes**

Transcriptomic resources can serve as an important source for the identification of valuable genetic markers like microsatellites, SNPs, etc. using computational approaches thus
avoiding costly and time consuming genome sequencing. We utilized whole genome transcriptome information for the identification of SSRs, which are useful in studying genetic diversity as well as DNA diagnostics purpose. Recently, SSR markers have been used to study genetic diversity in *Picrorhiza kurroa* [129] and also for authentication of *Picrorhiza kurroa* by discriminating it from its adulterant *Lagotis cashmiriana* [219]. It was reported that *P. kurroa* is considered genuine only when the motif (AAG)$_{17}$ targeting locus (EU883611) is amplifiable in a PCR reaction. The (AAG)$_{17}$ motif could not amplify in the morphologically similar adulterant species *Lagotis*.

Variable and uneven distribution of different types and abundance of repeat motifs has been observed in different plants [220]. It was observed that trinucleotide repeats were the most abundant repeats in *Picrorhiza kurroa*. Gahlan et al. [32] has also reported higher abundance of trinucleotide SSRs in *P. kurroa* transcriptome. Microsatellite sequences were successfully isolated from enriched genomic libraries of *Picrorhiza kurroa* by magnetic capture of microsatellite motifs [128] but are yet to be utilized for the assessment of genetic diversity. The majority of the microsatellites isolated were trinucleotide and dinucleotide repeats. The higher abundance of trinucleotide repeats has also been observed in various plants including wheat, cereal, grape, etc. [221, 222]. An absence of frameshift mutations due to the variety of trinucleotide repeats may be responsible for the high frequency of trinucleotide repeats in exonic regions [223]. Trinucleotide SSRs within exons may encode expressed amino acid runs. The majority of trinucleotide SSRs in exonic regions can be attributed to the suppression of non-trinucleotide SSRs in coding regions due to the risk of frameshift mutations, which might occur with non-trinucleotide microsatellites [223-226]

5.9 SSRs polymorphism in *P. kurroa* accessions

A low level of polymorphism was observed in *P. kurroa* accessions because the SSRs from transcriptomes were utilized in our study. SSRs in coding regions are more conserved than in genomic regions which limits SSR variation and tend to show less polymorphism in comparison to genomic SSRs [227]. SSR expansions and/or contractions in protein-coding regions can lead to a gain or loss of gene function via frameshift mutation or expanded toxic mRNA [225]. SSR variation in coding regions may also cause phenotypic changes and it has been speculated that organisms incorporating more DNA repeats might provide a molecular device for faster adaptation to environmental stresses [228, 229]. The SSRs with putative functions may be located in gene or regulatory regions. It was analysed that whether
SSRs were present in the regions of picrosides biosynthetic pathway genes by seeing the transcriptomic annotation of SSR regions. The location of specific SSRs in known genes and regulatory regions permits the unraveling of the biological significance of SSR distribution, expansion, and contraction on the function of the genes themselves. Though SSRs were not present in pathway genes, but it was observed that some of the polymorphic SSR markers were lying in important regulatory regions such as LRR receptor like serine/threonine protein kinase, WRKY transcription factor, translation initiation factor eif4a and primary metabolism like phosphofructokinase. Leucine-rich repeat receptor kinases (LRR-RKs) regulate a wide variety of developmental and defense-related processes in plants [230]. WRKY transcription factors are reported to act as repressors as well as activators in important plant processes [231] but most notably in coping with diverse biotic and abiotic stresses [232]. Translation initiation factors serve as the main regulatory element of the bottleneck of protein expression and are reported to be involved in plant abiotic stress tolerance [233]. Phosphofructokinase is a kinase enzyme that phosphorylates fructose 6-phosphate in glycolysis, the primary metabolic pathway providing precursors for picrosides biosynthesis. It is a key regulatory step of glycolytic pathway and is involved in a wide variety of biological processes [234]. All these genic regions might be responsible for regulation of picrosides biosynthesis and variation in P. kurroa. However, these studies need to be fully validated through functional analysis of genic regions in the context of picrosides content.

5.10 Genetic diversity analysis and clustering of P. kurroa accessions

The study indicated that P. kurroa populations in the North-Western Himalayan region are not genetically highly diverse at SSR loci as low level of polymorphism was observed in P. kurroa accessions. This plant is self-regenerating which propagates mostly through stolons by asexual reproduction as seed viability is very less [46, 2]. It prefers cross-pollination but self-pollination also occurs to some extent [46]. This indicates that P. kurroa might have a mixed mating system i.e. cross pollination by pollen and seed dispersal as well as selfing through rhizomes. Low genetic diversity indicates inbreeding which occurs when individuals with similar genotype are more likely to mate with each other rather than individuals with different genotypes which can lead to a reduction in genetic variation. It has been reported in various studies that there is low genetic diversity in endangered plant species. As P. kurroa is an endangered medicinal herb low genetic diversity in our case is justified and supported by various examples. Low genetic diversity has been reported in
critically endangered *Omphalogramma souliei* (Primulaceae) [235], *Dysosma versipellis* (Berberidaceae) [236] and *Glyptostrobus pensilis* (Cupressaceae) [237]. It has also been stated by Hamrick and Godt [238] and Frankham [239] that endangered plant species show lower genetic diversity than other species. Though a recent study done by Katoch et al. [129] claimed high genetic variation in twenty five *P. kurroa* accessions collected from four states (Himachal Pradesh, Uttarakhand, Jammu & Kashmir and Manipur) using limited number of SSR primers designed from rice genomic region. But genetic diversity assessment was not done in relation to picrosides contents in those accessions. In our study we have utilized a significantly high number of SSR primers designed from transcriptomes for more appropriate assessment of genetic diversity. The genetic diversity results in our study are not in accordance with that done by Katoch et al. [129] which might be due to the utilization of SSRs from different genic regions. Low genetic diversity in our study is contributed to the SSRs been utilized from transcriptomic regions rather than genomic region. However, our results are in agreement with the previous reports on various plant species which show that genomic SSRs revealed higher polymorphism and genetic diversity in comparison to EST or coding region derived SSRs [240-243]. This suggests that using different marker systems like SSRs from coding as well as non-coding regions could be more useful in genetic diversity studies in plant species having narrow genetic base [242].

To determine genetic relationships between *P. kurroa* accessions, cluster analysis was done using two methods i.e Structure and DARwin and the results obtained through both the methods were comparable. The accessions were grouped into two clusters according to picrosides content range but there were few exceptions in both the clusters which were not in accordance with picrosides content range. Our results are in accordance with the study done in *Hypericum perforatum* where partial correlation between hypericin content and SSR data has been observed in cluster analysis and it was reported that differences in genetic profile among accessions of the same species were not highly significant [244]. The importance of cluster analysis is that high content accessions from different geographical locations which are genetically similar can serve as a substitute for one another if any location is unapproachable due to unfavourable environmental conditions.