BIOSYNTHETIC MACHINERY OF IRIDOID GLYCOSIDES - THE MAJOR PHARMACOLOGICAL COMPONENTS OF A MEDICINAL HERB *PICRORHIZA KURROA* ROYLE EX. BENTH

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

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By

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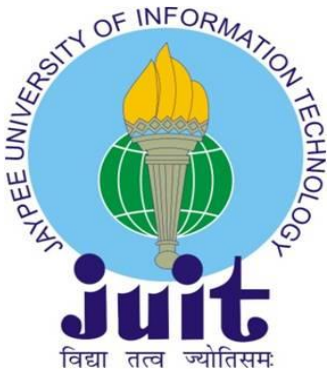

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CERTIFICATE

This is to certify that the thesis entitled, **"Biosynthetic Machinery of Iridoid Glycosides - The Major Pharmacological Components of a Medicinal Herb *Picrorhiza kurroa* Royle ex. Benth"** which is being submitted by **Kirti Shitiz (Enrollment No. 106560)** in fulfillment for the award of degree of **Doctor of Philosophy** in **Biotechnology** at **Jaypee University of Information Technology, Waknaghat, India** is the record of candidate's own work carried out by him under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Kirti Shitiz

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LIST OF ABBREVIATIONS

10HD	10-hydroxygeraniol dehydrogenase
2HFD	2-hydroxyisoflavanone dehydratase
ACT	Anthocyanin acyltransferase
ACTH	Acetoacetyl-CoA thiolase
ALD	Aldehyde dehydrogenase
APD	Arogenate/prephenate dehydratase
C4H	Cinnamate 4-hydroxylase
CAM	Caffeic acid-3-o-methyltransferase
CDP-ME	4-diphosphocytidyl-2-C-methylerythritol
CM	Chorosmate mutase
CME-PP	2-C-methyl-D-erythritol 2,4-cyclopyrophosphate
CPM	Cytochrome P-450 monooxygenase
CS	Chorismate synthase
DAHPS	3-Deoxy-D-arabinoheptulosonate 7-phosphate synthase
DCH	Deacetoxycephalosporin-C hydroxylase
DMAPP	Dimethylallyl pyrophosphate
DOXP	1-Deoxy-D-xylulose 5-phosphate
DQD	Dehydroquinate dehydratase
DQS	Dehydroquinate synthase
DSD	Dehydroshikimate dehydratase
DXPS/R	1-deoxy-D-xylulose 5-phosphate synthase/reductase
EPSPS	5-enolpyruvylshikimic acid-3-phosphate synthase

F3D	Flavanone 3-dioxygenase
FPKM	Fragments Per Kilobase Of Exon Per Million Fragments Mapped
G10H	Geraniol 10-hydroxylase
GDPS	Geraniol diphosphate synthase
GS	Geraniol synthase
HDS	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase
HMB-PP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMGS/R	Hydroxymethyl glutaryl CoA synthase/reductase
IPPI	Isopentenyl pyrophosphate isomerase
IS	Iridoid Synthase
MC	Monoterpene Cyclase
ISPD	2-C-methylerythritol 4-phosphate cytidyl transferase
ISPE	4-(cytidine-50-diphospho)-2-Cmethylerythritol kinase
ISPH	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase
MECPS	2-C-methylerythritol-2,4-cyclophosphate synthase
MEP	2-C-methyl-D-erythritol 4-phosphate
MVDD	Mevalonate diphosphate decarboxylase
MVK	Mevalonate kinase
NGS	Next generation sequencing
PAL	Phenylalanine ammonia lyase
PIC	Polymorphism Information Content
PMK	Phosphomevalonate kinase

QSD	Quinate/shikimate dehydrogenase
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
SK	Shikimate kinase
SQM	Squalene monooxygenase
SSR	Simple Sequence Repeat
TAT	Tyrosine aminotransferase
UGD	Uridine diphosphate glucuronic acid decarboxylase
UGT	Uridine diphosphate glycosyltransferase
UPD	Uroporphyrinogen decarboxylase

ABSTRACT

Picrorhiza kurroa possesses a broad range of pharmacological activities which are attributed to the presence of iridoid glycosides, Picroside-I (P-I) and Picroside-II (P-II). *P. kurroa* has been collected recklessly for its underground parts from the wild as a result putting it in the category of endangered species. Herbal drug industries purchase raw plant material (dried rhizomes) of *P. kurroa* from markets on the basis of physical appearance of the rhizomes rather than their chemical constituents. The biosynthetic machinery of major medicinal components, Picroside-I and Picroside-II is poorly understood. Thus any genetic intervention strategy for enhanced production of picrosides would require knowledge on complete biosynthetic pathway and key control points. The current study, therefore, investigated (i) quality assessment of *P. kurroa* raw material from major herbal drug markets of North-India; (ii) determination of chemotypic variation among accessions from different geographical locations; (iii) mapping genes to enzymatic steps in complete biosynthetic pathway of Picroside-I and Picroside-II; (iv) discerning the relative contribution of MEP/MVA pathway to the biosynthesis of Picroside-I and Picroside-II; and (iv) determination of genetic variation among *P. kurroa* accessions from different geographical locations.

Quality assessment of plant material form major herbal drug markets revealed that rhizomes sold in the Amritsar market contained highest picrosides content of 10.9% whereas sample from China (sold in Delhi market) showed the least amount of picrosides (2.8%). The rhizomes of twenty six accessions of *P. kurroa* showed a variation ranging from 2.9% to 10.9% in total picrosides content. Picroside-I content ranged from 0.38% to 2.7% in fresh shoots of the same accessions. The complete biosynthetic pathway of Picroside-I and Picroside-II was deciphered. Retrieval of gene sequences for enzymatic steps from transcriptomes and their expression analysis vis-à-vis picrosides content in different tissues/organs showed elevated transcripts for twenty genes, which were further shortlisted to seven key genes, ISPD, DXPS, ISPE, PMK, 2HFD, EPSPS and SK with ~5-57 folds transcript abundance on the basis of expression analysis between high versus low picrosides content strains of *P. kurroa*. The major contribution of MEP pathway in picrosides biosynthesis was ascertained through enzyme inhibitors which resulted in 17%-92% inhibition of P-I accumulation. Further, molecular characterization of twenty six *P. kurroa*

accessions using simple sequence repeat (SSR) markers showed that out of 360 SSRs tested, 35 yielded polymorphic profiles.

This study highlights the importance of quality assessment of *P. kurroa* raw material being used in the preparation of herbal drug formulations. The genetically superior accessions of *P. kurroa* can be recommended for mass cultivation to provide uniform high quality raw material. Key genes contributing to picrosides biosynthesis have been identified with potential implications in molecular breeding and metabolic engineering of *P. kurroa*. The information on SSR markers would be further helpful in the development of DNA diagnostics for the authentication of quality plant material.

CHAPTER 1

INTRODUCTION

Picrorhiza kurroa also known as Kutki is a small perennial herb from Scrophulariaceae family found in the Himalayan region at an altitude of 2500-4500m. It is mainly valued for its hepatoprotective activity as well as other medicinal properties like anti-malarial, anti-inflammatory, anti-oxidant, anti-bacterial, immune modulator, etc. which are attributed to the presence of iridoid glycosides, Picroside-I and Picroside-II [1]. It also contains apocynin, a powerful anti-inflammatory agent, which reduces platelet aggregation. Although *P. kurroa* is self-regenerating, however indiscriminate collection of its underground parts for extraction of active constituents has led to considerable depletion of natural populations [2]. Also the seeds have low viability [3] and as a result, the plant is now listed as an endangered species by International Union for Conservation of Nature and Natural Resources [4] and has been included in the Appendix II of CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) list.

Herbal drug formulations have been an integral part of Ayurvedic system of medicine for centuries. 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 present in desired concentrations [5]. *P. kurroa* is used in a number of commercially available herbal drug formulations like livocare, livomap, livplus, katuki, arogya, etc. for different disorders containing combinations of Picroside-I and Picroside-II in different concentrations (Table 1.1, Bhandari et al. [1]). Picroside-I and Picroside-II possess different medicinal properties individually as well as in combination and are therefore, two essential constituents of *P. kurroa* having therapeutic importance in several herbal drug formulations [6]. Picroside-I is reported to be anti-cancerous against human breast cancer [7] and used against hepatitis B [8, 9]. Picroside-II possesses different properties such as antiapoptotic [10], neuroprotective [11], anti-inflammatory [12], anti-oxidant [13] and prevents myocardial ischemia reperfusion injury [14]. The proper concentration and ratio of Picroside-I and Picroside-II are, therefore, important in determining the quality and efficacy of *P. kurroa*-based herbal drug formulations. A drug named picroliv has been developed by Central Drug Research Institute (CDRI), Lucknow,

India for acute and chronic hepatitis containing 60% of 1: 1.5 mixture of picroside-I and kutkoside [15].

Table 1.1 Herbal drug formulations based on *P. kurroa* (Bhandari et al. [1])

Formulation	Picroside-I (%)	Picroside-II (%)	Total (%)
Katuki, Zandu Pharma Works Ltd. (Mumbai, India)	1.29	1.16	2.45
Arogya, Zandu Pharma Works Ltd. (Vapi, GJ, India)	1.01	0.55	1.56
Kutaki, Tansukh Herbal Pvt. Ltd. (Lucknow, UP, India)	4.17	3.25	7.42
Livocare, Dindayal Aushdi Pvt. Ltd. (Gwalior, MP, India)	0.06	0.14	0.20
Livomap, Maharishi Ayurveda Pvt. Ltd. (New Delhi, India)	0.12	0.06	0.18
Livomyn, Charka Pharma Pvt. Ltd. (Mumbai, India)	0.07	0.01	0.08
Livplus, BACFO Pharmaceuticals Ltd. (Noida, UP, India)	0.22	0.49	0.71
Pravekliv, Pravek Kalp Herbal products Pvt. Ltd. (Noida, UP, India)	0.15	0.12	0.27
Vimliv, OM Pharma Ltd. (Bangalore, India)	0.001	Traces	0.001

The increasing demand of *P. kurroa* plant material for preparation of herbal drugs necessitates the screening of natural populations for identification of superior chemotype (genotypes) with desirable amount of picrosides. A chemotype can be considered as superior if it is having desired amount of chemical constituents which are uniformly present across different environments. Herbal drug industries are looking for authentic plant material with desired amounts of active compounds. Increasing demand for kutki has prompted many researchers to search for sources of genotypes of *P. kurrooa* rich in picroside content [16]. Considerable variation in picrosides content has been observed in different populations of

P. kurroa [16-18], however, all these studies quantified Picroside-I and Picroside-II in rhizomes of populations collected from different geographical locations where contents are influenced not only by the genetic makeup but also due to age and developmental stage of rhizomes, in addition to altitude, temperature and other environmental factors. These factors play an important role, as the uniformity in the selection of plant material is essential for the preparation of herbal drug formulations. Inspite of the medicinal importance and variation in picrosides content in natural populations no attempt has been made to characterize genetic variation and population structure in natural populations of *P. kurroa* vis-à-vis Picroside-I and Picroside-II contents. Moreover, the analysis of genetic diversity and relatedness between or within different genotypes is a prerequisite toward efficient utilization and protection of plant genetic resources [19, 20]. Also, the study of genetic diversity would be helpful in the development of DNA diagnostics for the authentication of superior genotypes. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in plants and also, the detection of genes influencing economically important traits [21].

The biosynthesis and accumulation of Picroside-I and Picroside-II occur differentially in different tissues of *P. kurroa*. Picroside-I is produced predominantly in shoots and Picroside-II in roots or stolons whereas both accumulate in rhizomes [22, 23]. The precursors for the biosynthesis of majority of terpenoid class of secondary metabolites are formed through common routes, cytosolic mevalonate pathway (MVA) and plastidic non-mevalonate (MEP) pathway. Biosynthesis of picrosides occurs through a combined biosynthetic route involving non-mevalonate (MEP), mevalonate (MVA), phenylpropanoid and iridoid pathways. Picrosides are monoterpenoids with iridoid backbone and glycoside moiety. 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 isomer dimethylallyl diphosphate (DMAPP) via cytosolic mevalonate (MVA) and/or plastidic (MEP) pathway [25, 26]. Biosynthesis of picrosides involves the synthesis of iridoid moiety from GPP through series of oxidation and cyclization steps followed by condensation of glucose moiety and cinnamate/vanillate from phenylpropanoid pathway [27, 28]. A schematic representation of Picroside-I and

Picroside-II biosynthetic pathway adapted from Mahmoud and Croteau [29] is shown in Figure 1.1.

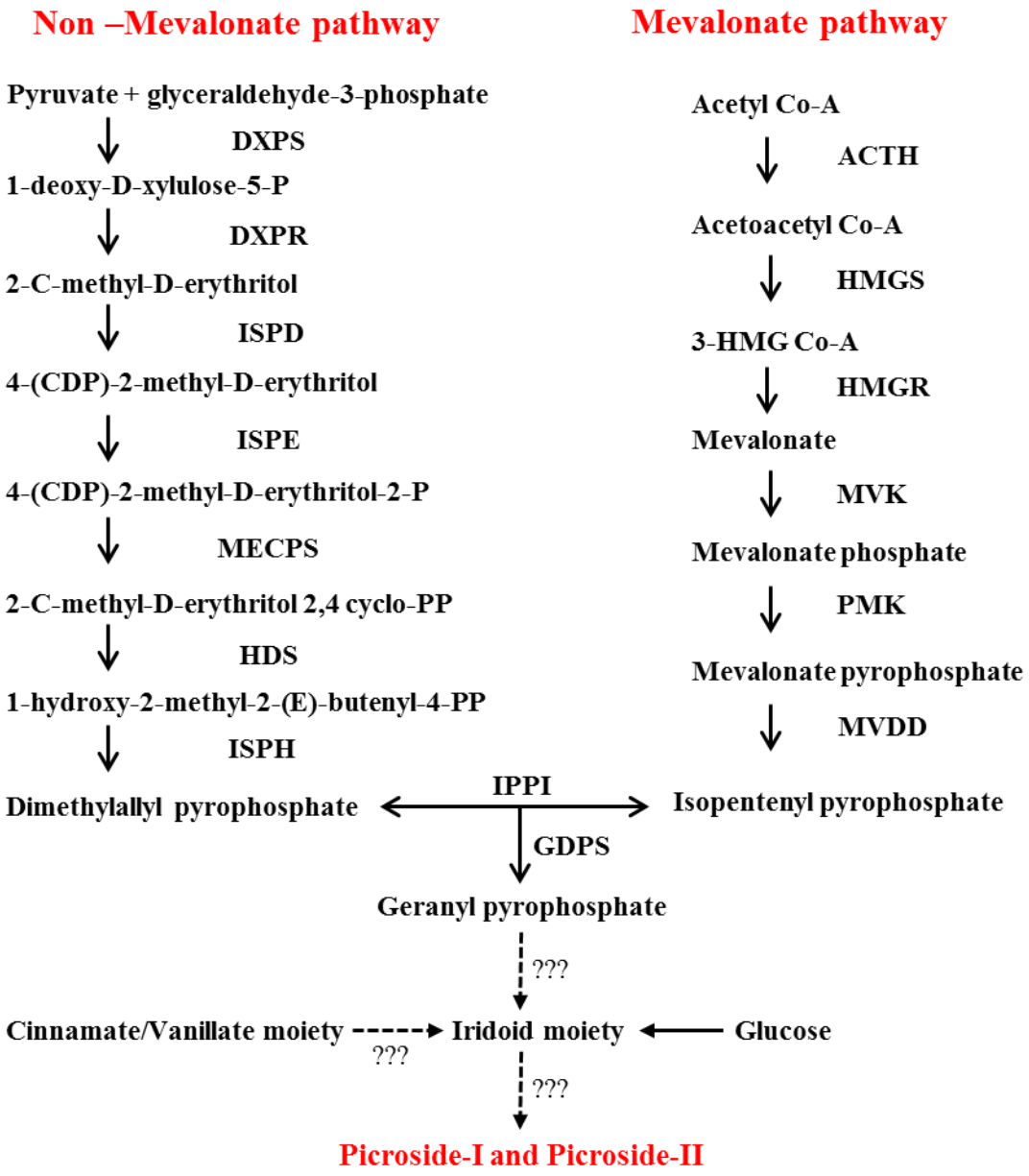


Figure 1.1 Schematic pathway for Picroside-I and Picroside-II biosynthesis (adapted from Mahmoud and Croteau [29])

The complete biosynthetic pathway of picrosides has been deciphered for all possible intermediates [28], however, the identity, role and contribution of corresponding genes catalysing the enzymatic steps is not known. There are 41 steps (15 from MEP and MVA pathway, 14 from iridoid pathway, 11 from phenylpropanoid pathway, 1 involved in esterification of catalpol) and 35 steps (15 from MEP and MVA pathway, 14 from iridoid

pathway, 5 from phenylpropanoid pathway, 1 involved in esterification of catalpol) involved in the biosynthesis of Picroside-I and Picroside-II, respectively. The cinnamate/vanillate moieties are first CoA activated and then transferred to the catalpol for the formation of respective iridoids. Out of 35, 32 steps till 3-dehydroshikimate are common for both Picroside-I and Picroside-II. After that Picroside-II pathway is diverted for the production of vanillic acid (4-hydroxy-3-methoxybenzoic acid) and Picroside-I pathway is diverted for the production of cinnamic acid. The final step involved in the esterification of catalpol (iridoid backbone) for the final biosynthesis of Picroside-I and Picroside-II is also common. An alternative route for the formation of vanillic acid by degradation of ferulic acid has also been reported in *Vanilla planifolia* [30] and *Pseudomonas fluorescens* [31]. Various studies have reported partial biosynthetic pathway for picrosides along with cloning and characterization of few individual enzymatic steps. Kawoosa et al. [27] reported MEP and MVA pathways for picrosides biosynthesis adapted from Mahmoud and Croteau [29] which are common for biosynthesis of all terpenoids but the specialized pathway from GPP till the formation of picrosides was incomplete. Additionally, rate limiting enzymes of MEP pathway (DXPS) and MVA pathway (HMGR) were cloned in *P. kurroa*. Two genes of phenylpropanoid pathway (4-CH and 3-CH) and involvement of CYPs and glycosyltransferases in picrosides biosynthesis was also reported [32]. Singh et al. [24] cloned 8 genes of the MEP and MVA pathways and reported two additional genes (PAL and COMT) of phenylpropanoid pathway. Five remaining genes of MEP and MVA pathway were cloned by Pandit et al. [33]. Cloning of UGT gene of iridoid pathway was done by Bhat et al. [34]. Each of these studies has shown that the studied enzymatic steps are playing important role in the biosynthesis of Picroside-I and Picroside-II. However, none has clarified as to which of the MVA/MEP pathways contribute to the iridoid backbone, GPP and which genes are playing key role in the biosynthesis of Picroside-I and Picroside-II in *P. kurroa*.

Multiple pathways contribute to the biosynthesis of secondary metabolites, therefore, it is necessary to identify the flux of individual pathways for the production of final product. Inhibitor studies can provide significant clues in ascertaining the contribution of individual biosynthetic pathways towards production of terpenoids. Palazon et al. [35] showed that non-mevalonate pathway is the main source of universal terpenoid precursor isopentenyl diphosphate (IPP) for the biosynthesis of taxanes in *Taxus baccata*. The major involvement of mevalonate pathway for shikonins biosynthesis in *Arnebia euchroma* has also been

proved through inhibitor assays [36]. However, no such effort has been made in *P. kurroa* for the identification of which pathway module contributes to the biosynthesis of Picroside-I and Picroside-II.

The biosynthesis and accumulation of medicinally important metabolites 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 formulations. Differential conditions for the biosynthesis and accumulation of chemical constituents in medicinal herbs are not only suitable for understanding the biology of their biosynthesis, including biosynthetic pathways but also in regulating the quality of herbal drugs. Conditions with differential contents of Picroside-I and Picroside-II are suitable to associate and ascertain the role and contribution of pathway genes towards their biosynthesis.

Current research work focused on quality assessment of *P. kurroa* plant material from herbal drug markets of North India along with assessment of variation in major chemical constituents (Picroside-I and Picroside-II) across a collection of populations of *P. kurroa* which belonged to different geographical locations of North-Western Himalayas (Himachal Pradesh) but planted at a common location so as to reflect on whether variation in Picroside-I and Picroside-II contents is due to genetic factors. We generated NGS transcriptomes of six *P. kurroa* tissues having differential biosynthesis and accumulation of Picroside-I and Picroside-II as transcriptomic resources can be helpful in accelerating gene discovery and annotation, expression analysis, identifying splice variants, and identification of molecular markers such as SNPs, SSRs, etc. To identify the molecular basis of picrosides biosynthesis and variation at gene level, expression analysis of all genes of the biosynthetic pathway, after mapping unknown genes of the phenylpropanoid and iridoid branch, was carried out in four different tissues of *P. kurroa* with contrasting contents of Picroside-I (0.0 % and 2.7%) and Picroside-II (0.0% and 4.0%). To ascertain the involvement of key genes in picrosides biosynthesis, expression analysis was also done on high versus low Picroside-I content accessions. Further, we ascertained the contribution of MVA and/or MEP route and importance of other integrating pathways in the biosynthesis of picrosides through enzyme inhibitor experiments. The complete biosynthetic pathway of picrosides, including mapping of all genes catalyzing different enzymatic steps has been deciphered for the first time in *P. kurroa*. Finally, molecular characterization of twenty six *P. kurroa* accessions varying for Picroside-I and Picroside-II contents was carried out. Simple sequence repeat (SSR)

markers were utilized for this purpose which have been widely used in plant germplasm evaluation. The availability of transcriptomes of *P. kurroa* enabled us to mine for whole SSRs complement and then to utilize in characterization of genetic diversity vis-à-vis picrosides content.

Keeping in view the medicinal importance of *P. kurroa*, lack of genetic basis for variation in picrosides content in *P. kurroa* accessions and incomplete knowledge on biosynthetic machinery of picrosides, the current research work was carried out with following objectives:

- Quality assessment of *P. kurroa* plant material from major herbal drug markets of North-India and determination of chemotypic variation among accessions from different geographical locations
- Mapping genes to enzymatic steps in complete biosynthetic pathway of Picroside-I and Picroside-II in P. *kurroa*
- Discerning the relative contribution of MEP/MVA pathway to the biosynthesis of Picroside-I and Picroside-II in *P. kurroa*
- Determination of genetic variation among *P. kurroa* accessions from different geographical locations

CHAPTER 2

REVIEW OF LITERATURE

Natural products have been used since ancient times for the treatment of various ailments and disorders. Natural products are chemical compounds or substances produced by living organisms usually plants, found in nature that usually have pharmacological or biological activity. Plants produce a vast and diverse assortment of chemical compounds, the metabolites, which are present as structurally different compounds across various species. Secondary metabolites have a wide array of uses ranging from flavouring agents to medicinal value. They are derived from the precursors synthesized by fundamental or primary metabolic pathways. Secondary metabolites are categorized into following categories namely, terpenoids (derived from acetyl coenzyme A or glycolysis cycle intermediates), alkaloids (nitrogen containing compounds), glycosides, natural phenols (aromatic rings bearing a hydroxyl functional group), phenazines, polyketides, fatty acids, etc. Terpenoids are further classified into sub-categories depending on the number of isoprene units (C_5). Monoterpenes (C_{10}) made from two isoprene units, sesquiterpenes (C_{15}) made from three isoprene units, diterpenes (C_{20}) made from four isoprene units, triterpenes (C_{30}) made from six isoprene units and tetreterpenes (C_{40}) made from eight isoprene units.

Secondary metabolites are differentially distributed among limited taxonomic groups within the plant kingdom [37]. The natural variation of these secondary metabolites is more extensive quantitatively and qualitatively [38]. The medicinal plants are highly valued for their secondary metabolites required for the formulation of herbal drugs. A growing concern with the awareness of side effects of synthetic drugs has triggered a “back-to-nature” idea with an appeal of new discovery of natural products [39]. Therefore, the demand of high-yield/high-quality medicinal plants will continue to increase in future.

In order to gain a better understanding of the genetic regions that control secondary metabolite production, it is necessary to study genetic variation and identify loci or genes linked with high content of secondary metabolites. 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 secondary metabolite production and

variation is suggested to be regulated by multiple genes. Understanding the process of biosynthesis and accumulation of secondary metabolites requires identification of genes/pathways involved in the biosynthesis and studying their expression patterns in relation to secondary metabolites contents. Genomics and/or systems approaches integrating transcript profiling with metabolite measurements have facilitated the study of genetic mechanisms underlying secondary metabolism. These approaches can help identify uncharacterized networks or pathways, in addition to candidate regulators of such pathways [41].

2.1 *Picrorhiza kurroa*

Picrorhiza is a small genera belonging to the tribe Veroniceae of the family Scrophulariaceae. This family is, according to the taxonomical system of Cronquist, arranged in the order Scrophulariales, subclass Asteridae, class Dicotyledonae, of the Angiospermae. *Picrorhiza* which is used as a native medicine, has derived the name from its bitter root [42]. In Greek, 'picros' means bitter, while 'rhiza' means root. *Picrorhiza kurroa* was first identified by Smith and Cave [43] at the base of the Zemu glacier in Sikkim at a height of 4,300 m. Two species of *Picrorhiza* have been characterized, *P. kurroa* located in the dry Western Himalayas having long stamens, while *P. scrophulariiflora* is the species located in the moist Eastern Himalayas having short stamens [44]. Recently, a third species *Picrorhiza tungnathii* has been identified in Western Himalayas from Uttarakhand, India [45].

Picrorhiza kurroa is a perennial herb which propagates 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 and apparently separate looking plants at above ground level are actually joined together by stolons beneath (Figure 2.1, Raina et al. [46]). However, when these stolons get detached from the mother stock, the plants become independent. It has a long, creeping rootstock that is bitter in taste, and grows in rock crevices and moist, sandy soil. The leaves of the plant are flat, oval, and sharply serrated. The flowers, which appear June through August, are white or pale purple and borne on a tall spike; manual harvesting of the plant takes place October through December. It prefers cross-pollination but self-pollination also occurs to some extent. P. kurroa is diploid (2n=34) having estimated genome size of 3452.34 Mbp [47].

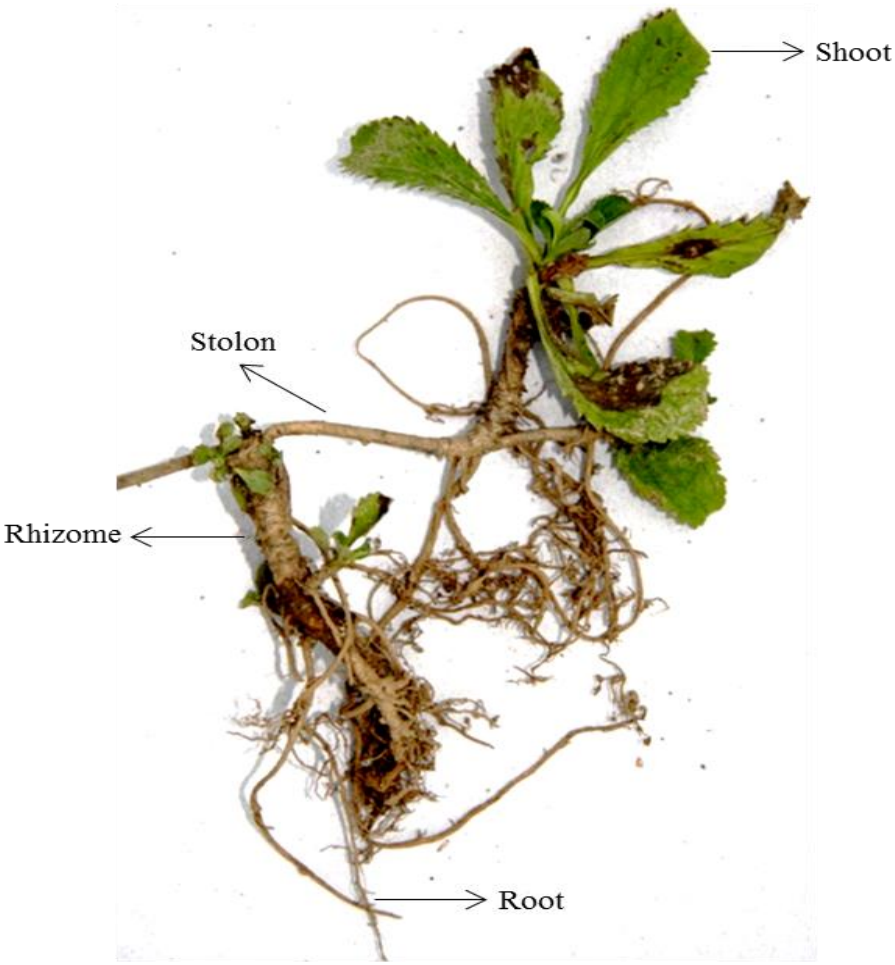


Figure 2.1 Mature *P. kurroa* plant showing different organs

2.2 Phytochemistry of *P. kurroa*

The major chemical constituents of *P. kurroa* have been obtained from roots and rhizomes. Kutkin is the active principal of *Picrorhiza kurroa* and is comprised of kutkoside and the iridoid glycoside picrosides I, II, and III. Other identified active constituents are apocynin, drosin, and nine cucurbitacin glycosides [48, 49]. Picroside-I and Picroside-II are the two important iridoid glycosides of *P. kurroa* (Figure 2.2). The major classes of chemical compounds isolated from *Picrorhiza* species are iridoid glycosides, cucurbitacins, phenolic and phenylethanoids (Table 2.1).

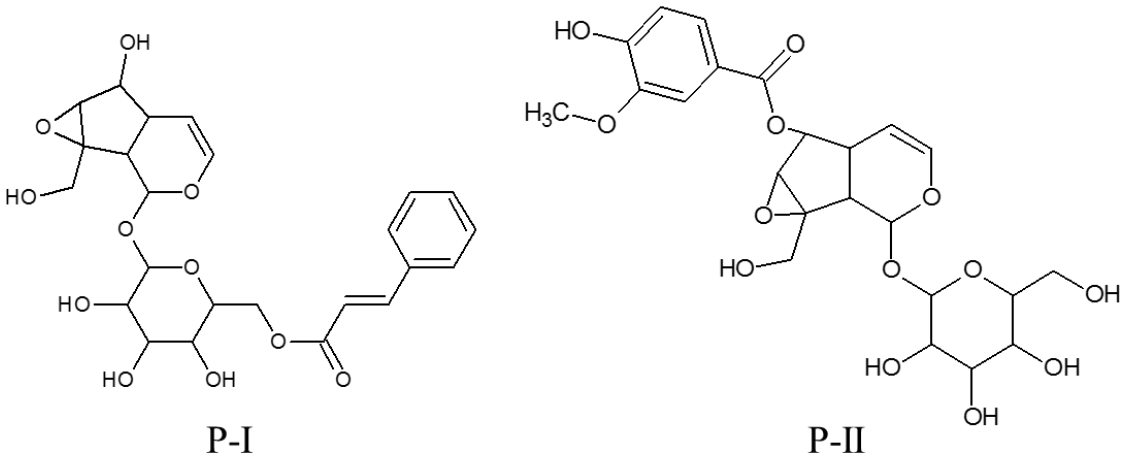


Figure 2.2 Chemical structures of Picroside-I and Picroside-II

Table 2.1 Major chemical constituents isolated from *Picrorhiza* species

Class	**Compound**	**Reference(s)**
Iridoids	Picroside-I	Kitagawa et al. [50]
	Picroside-II	Weinges et al. [48]
	Picroside-III	Weinges and Künstler [51]
	Picroside-IV	Li et al. [52]
	Picroside-V	Simons [53]
	Veminoside	Li et al. [52]
	Catalpol	Wang et al. [54]
	Veronicoside	Stuppner and Wagner [49]
	Specioside	Li et al. [52]
	6-feruloylcatalpol	Stuppner and Wagner [49] Simons 1989 [53]
	Pikuroside	Jia et al. [55]
	Aucubin	Wang et al. [54]
	Kutkoside	Singh and Rastogi [56]
	Minecoside	Stuppner and Wagner [49] Simons [53]
Cucurbitacins	Arvenin I	Stuppner and Wagner [49]

	Arvenin II	Stuppner and Wagner [49]
	Arvenin III	Stuppner et al. [57]
Phenolics	Vanillic acid	Rastogi et al. [58]
	Apocynin	Basu et al. [59]
	Androsin	Wang et al. [54]
	Picein	Stuppner and Wagner [49]

2.2.1 Iridoid glycosides

Medicinally important iridoid glycosides have been isolated and characterized from *Picrorhiza*; Picroside I [50], Picroside II [48, 54], Picroside III [51], Picroside IV [52], Picroside V [53], Verminoside [52], Catalpol [54], Veronicoside [49], Specioside [52], 6-feruloylcatalpol [53, 49], Pikuroside [55], Aucubin [54], etc. These iridoids belong to the family of terpenoids which has more than 30,000 members possessing important biological and physiological functions in plants [60]. Kutkin and Picroliv are the main herbal preparations of *P. kurroa*; Kutkin is a mixture of picroside I and kutkoside in a ratio of 1:2 and other minor glycosides [56, 61] whereas Picroliv is a similar but less purified fraction, containing about 60 % of an equal mixture of Picroside-I and kutkoside [62].

2.2.2 Cucurbitacins

These are triterpenoid compounds that are both bitter and toxic. Cucurbitacins possess a wide range of biological activities and are present in the form of β-glycosides in plants [63]. A total of 23 cucurbitacin glycosides and one aglucone has been isolated from *Picrorhiza* species mainly from *P. kurroa* [64].

2.2.3 Phenolics

They are precursors and degradation products of lignin, which provide sturdiness to the plant and a physical defense barrier against parasites [65]. Various phenolics isolated from *P. kurroa* are vanillic acid [58], apocynin [59], androsin [54] and picein [49].

2.3 Medicinal value of *P. kurroa* extract

Picrorhiza has been used in the traditional as well as modern systems of medicine. It is traditionally used for liver disorders, but has also been implicated in the treatment of upper respiratory tract, fevers, dyspepsia, chronic diarrhea, and scorpion stings. The medicinal properties of *P. kurroa* extract are listed in Table 2.2. The major properties have been described below:

2.3.1 Hepatoprotective: Picroliv, the active constituent isolated from *P. kurroa* has been shown to possess hepatoprotective activity [66]. Alcohol fed rats produced alteration in selected serum (AST, ALT and ALP) and liver markers (lipid, glycogen and protein), reduced the viability of isolated hepatocytes (ex vivo), reduced the levels of alcohol-metabolizing enzymes (aldehyde dehydrogenase, acetaldehyde dehydrogenase) in rat hepatocytes and also produced cholestasis, as indicated by reduction in bile volume, bile salts and bile acids. After treatment with Picroliv all these altered parameters were restored. It has also proved to be effective against viral hepatitis in humans [67]. A hydroalcoholic extract of *P. kurroa* has been shown to be effective against non-alcoholic fatty liver disease (NAFLD) by reversal of the fatty infiltration of the liver and a lowering of the quantity of hepatic lipids [68].

2.3.2 Antioxidant: Rajkumar et al. [69] investigated the antioxidant activity of methanolic and aqueous extracts of *P. kurroa* rhizome. The antioxidant efficacy was studied using radical scavenging assays, ferric reducing antioxidant property and thiobarbituric acid assay for testing inhibition of lipid peroxidation. Antioxidant activity of leaf extract [70] and ethanol extract of rhizome on indomethacin induced gastric ulcer has been demonstrated [71].

2.3.3 Immunomodulatory: Numerous biopolymeric fractions have been isolated from medicinal plants and used as a source of therapeutic agents. The biopolymeric fraction RLJ-NE-205 isolated and purified from the rhizomes of *P. kurroa* was investigated and found to be immunomodulatory [72]. The effects of RLJ-NE-205 were evaluated on *in vivo* immune function of the mouse. Significant increases in the proliferation of lymphocytes and cytokine levels (IL-4 and IFN-gamma) in serum were observed. An increase was demonstrated in HA titre, DTH, PFC, phagocytic index and CD4/CD8 population. This suggests that the biopolymeric fraction RLJ-NE-205 improves the immune system and might be regarded as

a biological response modifier. RLJ-NE-205 has been evaluated for its potential ability as an adjuvant effect on the immune responses to Ovalbumin (specific antigen) in mice [73].

2.3.4 Antineoplastic and anticancerous: Antineoplastic activity of rhizome extracts of *P. kurroa* has been demonstrated by Rajkumar et al. [69]. The cytotoxicity was tested by XTT assay in MDA-MB-435S (human breast carcinoma), Hep3B (human hepatocellular carcinoma) and PC-3 (human prostate cancer) cell lines. Anti-tumour and anti-carcinogenic activity of *P. kurroa* extract has been demonstrated in mice [74]. Oral administration of *P. kurroa* extract inhibited the tumour incidence and tumour related deaths in sarcoma induced mice. The extract also reduced the volume of transplanted solid tumours, increased the life span of ascites tumour bearing mice and inhibited yeast topoisomerase I and II enzyme activity when tested on *Saccharomyces cerevisiae* mutant cell cultures.

2.3.5 Antimalarial and antimicrobial: Antimalarial potential of *P. kurroa* against *Plasmodium berghei* has been shown by Singh and Banyal [75]; Kumar and Sharma [76]. *P. kurroa* extracts were tested and found to be effective against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Aspergillus niger* and *Candida albicans* [77]. The protective effect of Picroliv has also been demonstrated against ulcerative colitis [78], hepatic amoebiasis [79], *Leishmania donovani* infections in *Mesocricetus auratus* [80] and *Mycobacterium tuberculosis* infections in humans [81]. The use of picroliv as adjunct to antileishmanial chemotherapy by enhancing antileishmanial efficacy and lymphocyte proliferation has been demonstrated [82].

2.3.6 Antidiabetic: Diabetes mellitus was induced with streptozotocinnicotinamide and rats found diabetic were orally administered standardized aqueous extract of *P. kurroa* for 14 days. In oral glucose tolerance test, oral administration of *P. kurroa* extract increased the glucose tolerance. *P. kurroa* extract treatment also reversed weight loss associated with streptozotocin treatment. These findings provided *in vivo* evidence that standardized extract of *P. kurroa* possess significant antidiabetic activity in streptozotocin-nicotinamide induced type-2 diabetes mellitus in rats [83].

Table 2.2 Pharmacological value of *P. kurroa* extract

Medicinal Property	**Reference(s)**
Hepatoprotective	Sinha et al. [84]
Antioxidant	Rajkumar et al. [69]
Antiallergic and antiasthamatic	Dorch et al. [85]
Immunomodulatory	Sane et al. [82]
Antimalarial	Irshad et al. [86]
Anti-inflammatory	Zhang et al. [87]
Anticancerous	Joy et al. [74]
Nerve growth enhancer	Li et al. [88]
Used against NAFLD	Shetty et al. [68]
Antineoplastic	Rajkumar et al. [69]
Antidiabetic	Husain et al. [83]
Anticholestatic	Verma et al. [89]

2.4 Medicinal value of individual metabolites

2.4.1 Picroside-I: There are limited reports on pharmacological properties of Picroside-I. Zhou [9] has demonstrated that Picroside-I can be effective in the treatment of hepatitis B. Picroside-I also showed significant therapeutic effect on jaundice, chronic liver injury induced by carbon tetrachloride, and enhanced humoral immune response. The anti-invasion activity of Picroside-I against MCF-7 cell lines (human breast cancer) has also been demonstrated [7]. It exhibited considerable cytotoxic potential by inhibiting MCF-7 cell invasion and migration and down-regulation of the expression of matrix metalloproteinases (MMPs) at mRNA and protein level. Suppression of inflammatory mediators was also observed.

2.4.2 Picroside-II: Several studies have reported pharmacological properties of Picroside-II, thereby highlighting major medicinal importance of Picroside-II. Picroside-II has been

shown to prevent cerebral ischemic injury in rats by reducing the content of free radical and enhancing the activity of antioxidase [90] and enhancing the activity of GSHPx [91]. The neuroprotective effect of picroside II via regulating the expression of myelin basic protein after cerebral ischemia injury in rats has been evaluated [11]. Picroside-II down-regulates the expressions of TLR4, NFκB and TNFα to inhibit apoptosis and inflammation induced by cerebral ischemic reperfusion injury and improves the neurobehavioral function of rats [12]. Anti-lipid peroxidation and protection of liver mitochondria by Picroside-II against injuries in mice with liver damage has been investigated [92] alongwith inhibition of hepatocyte apoptosis [93], neuronal apoptosis [94] and apoptosis induced by renal ischemia/reperfusion injury in rats [10]. Picroside-II also protects cardiomyocytes from hypoxia/reoxygenation induced apoptosis [95]. The medicinal properties of individual Picroside-I and Picroside-II compounds is given in Table 2.3.

2.4.3 Apocynin: It is a constituent of root extract of *P. kurroa* which possesses anti-inflammatory properties [96]. The effect of apocynin was investigated on the production of arachidonic acid-derived inflammatory mediators by guinea pig pulmonary macrophages. Apocynin is reported to inhibit arachidonic acid-induced aggregation of bovine platelets possibly through inhibition of thromboxane formation. Apocynin, was also found to be a potent inhibitor of the super oxide anion generating NADP oxidase in human neutrophil membrane [97].

2.4.4 Catalpol: Catalpol protects mice against renal ischemia/reperfusion injury which is the most common cause of acute kidney failure [98]. It acts via suppressing phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt)-endothelial nitric oxide synthase (eNOS) signalling and inflammatory responses. Anti-cancer, neuroprotective, anti-inflammatory, diuretic, hypoglycemic, and anti-hepatitis virus effects of catalpol from other plants have also been demonstrated [99]. Catalpol regulates cholinergic nerve system function through effect on choline acetyl-transferase and may have beneficial effect for the treatment of Alzheimer's disease [100].

Table 2.3 Major medicinal properties of Picroside-I and Picroside-II compounds

Chemical Constituents	Medicinal Properties	Reference(s)
Picroside-I	Anti-cancerous against human breast cancer	Rathee et al. [7]
	Used against hepatitis B	Zhou 2008 [9]
Picroside-II	Anti-oxidant	Yang et al. [90]
	Neuroprotective	Zhao et al. [11]
	Anti-inflammatory	Guo et al. [12]
	Anti-apoptotic	Wang et al. [10]
	Prevents myocardial ischemia reperfusion injury	Wu et al. [14]
	Prevents cerebral ischemic reperfusion injury	Guo et al. [12]
	Reduce NO (Nitric oxide) concentration and enhance GSHPx activity	Wang et al. [91]
	Protects cardiomyocytes from hypoxia	Meng et al. [95] Pei et al. [101]
	Inhibit neural apoptosis	Li et al. [94]

2.5 Quality parameters for herbal drugs in harvestable parts

With an ever-increasing use of herbal medicines worldwide and rapid expansion of the global market for these products, safety and quality of medicinal plant materials and finished herbal medicinal products have become a major concern for healthcare, pharmaceutical industries and the public. The variability of the constituents in herbs or herbal preparations due to genetic, cultural and environmental factors has made the use of herbal medicines more challenging than it necessarily has been. For instance, the availability of quality raw materials is frequently problematic as the active principles are diverse or may be unknown, and quality of different batches of preparation may be difficult to ascertain and control. In most countries, herbal products are launched into the market without proper scientific evaluation, and without any mandatory safety and toxicological studies. There is no effective machinery to regulate manufacturing practices and quality standards. A well-defined and constant composition of the active constituent is one of the most important

prerequisites for the production of a quality herbal drug. Given the nature of products of plant origin, which are not usually constant and are dependent on and influenced by many factors, ensuring consistent quality of products is vital for the survival and success of the industry [102]. Along with other factors such as geographical location and methods of collection and storage, environmental factors prevailing during different seasons of the year may affect the quality and uniformity of the chemical constituents and their concentrations in medicinal plants.

P. kurroa is one of the top 15 medicinal plant species traded in India in terms of economic value of traded materials [103]. Its principle compounds, Picroside-I and Picroside-II possesses a broad range of pharmacological activities, therefore, proper concentration and ratio of these compounds are important in determining the quality and efficacy of *P. kurroa*-based herbal drug formulations. There is not only an increasing demand for large quantity of raw material of this medicinal herb, but also of appropriate quality where active principles are present in desired concentrations. Majority of the plant material is collected from the wild for extraction of active constituents from underground parts i.e. roots and rhizomes where picrosides contents can be influenced by several factors such as environment (altitude, light intensity, soil conditions, etc.), genetic makeup and time of collection of plant material. Different batches of plant material may differ in raw material quality due to these factors. It is important to specify the optimum time of harvesting as concentrations of picrosides can vary during different seasons. Plant material collected in the months of July-August is supposed to have high moisture content and generally rated low while material collected in September is highly valued due to low moisture content [104]. Moreover, within a plant, there is also variation among different organs, developmental stages and environmental conditions as identified in our previous study [23]. Variation in quantity of active components may influence the therapeutic potential of medicinal plant therefore, uniformity in the selection of plant material is essential for the preparation of herbal drug formulation. The plant material is largely collected from the wild, cleaned, dried and transported to the herbal drug market and supplied to herbal drug industries. However, what parameters determine the quality of plant material is not known. It may be only the physical appearance (thickness of rhizomes, presence of dust or other unwanted material, etc.) rather than the contents of marker compounds being used as selection criteria of plant material. It is necessary to determine whether morphological appearance of rhizomes is an appropriate criteria for evaluating the quality of raw plant material since that is used by most of the industry buyers. Moreover, due to increasing industrial demand and high economic value

there are chances of adulteration with morphologically similar plant material which can lead to reduced efficacy of herbal formulations. Therefore, quality assessment of plant material available in herbal drug markets is necessary. No studies have reported on quality assessment of *P. kurroa* plant material being supplied to herbal drug industries which is a prerequisite for the production of *P. kurroa* formulations with high efficacy. As picrosides contents are influenced by altitudinal variation, chemotypic profiling of strains from different geographical locations having high amount of picrosides would result in identification of superior strains which can be utilized for preparation of high quality herbal formulations of *P. kurroa*.

2.6 Variation in major phytochemical constituents in *P. kurroa*

Increasing demand for *P. kurroa* plant material has prompted many researchers to search for sources of genotypes rich in picrosides, which do not exist in *P. kurroa* as of today. The herbal drug industries preparing herbal formulations from medicinal plants are not only looking for higher contents of chemical constituents but also particular proportions of desired chemical constituents, which are uniquely present in elite chemotypes. The requirement of particular concentration and composition of desired chemical constituents in a herbal drug formulation has been emphasized by Picroliv, which is a herbal formulation from *P. kurroa* and reported to contain a definite ratio (1:1.5) of Picroside-I and Picroside-II [89]. Therefore, screening of natural populations of *P. kurroa* from different geographical locations is necessary to assess the extent of variation in picrosides content so as to identify superior accessions.

Picroside I and II content in various plant parts of *P. kurroa* collected from different geographical locations with varying altitudes, viz. Sonemarg (2,740 m a.s.l.), Tangmarg (2,690 m a.s.l.), and Pulwama (1,630 m a.s.l.) in the North-Western Kashmir Himalayas was reported [105]. Picrosides content was found to be highest in populations collected from Sonemarg followed by Tangmarg and Puwama suggesting that picrosides accumulation is directly correlated with altitudinal change. The quantities of picrosides also varied spatially, being highest in rhizome followed by roots, inflorescence and leaves in the populations from all three locations. In another study, rhizomes of four accessions of *P. kurroa* from different locations in Uttarakhand namely, Lohagang pass, Roopkund, Dewal and Ghat were quantified [106]. Picroside I ranged from 2.74 – 4.91 % and Picroside II from 3.12 – 5.39%, with Lohagang pass having highest amount of picrosides. Kumar et al. [17] evaluated

eighteen accessions of *P. kurroa* for growth and picrosides accumulation pattern under field conditions at Chamba (Himachal Pradesh) located at an elevation of 2538 m during 2006 to 2010 and identified six accessions with higher picrosides content and vegetative growth. Picrosides content ranged from 0.08- 8.05% in leaves and from 0.52- 9.02% in rhizomes. This study revealed variation in growth and picrosides (Picroside-I and Picroside-II) content in different accessions of *P. kurrooa* collected from the North-Western Himalayas and grown at lower altitude. Sharma et al. [18] analysed rhizomes of seven accessions of *P. kurroa* from Himachal Pradesh for variation in picrosides content which ranged from 2.21-5.5%.

The variability of major phyto-constituents within the same specie at different altitudinal ranges indicate a significant relationship between the quality and quantity of active principle and the environmental factors such as different habitat and stress conditions of different geographical locations. The variation in quantity of active components may influence the therapeutic potential of medicinal plant. Therefore, for the cultivation of such medicinally important plants the factors such as growth, environment and storage conditions etc. should be standardized to obtain better quality so as to improve the therapeutic effects.

2.7 Molecular basis of picrosides biosynthesis

Picrosides are iridoid glycosides of monoterpene origin [63]. The biosynthesis and accumulation of terpenoids is controlled at the molecular level by structural and regulatory genes in different plant species [107]. Lack of understanding about the biology and molecular basis of Picroside-I and Picroside-II biosynthesis and accumulation impedes perusal of a systematic genetic improvement programme in *P. kurroa*. There have been limited efforts so far in understanding the molecular biology of picrosides biosynthesis in *P. kurroa*, including identification of genes/enzymes and metabolic pathways. No studies have reported the complete biosynthetic pathway for picrosides till date. The modern approaches of pathway elucidation and identification of missing steps in the biosynthetic pathways involve correlating the expressions of genes (at mRNA & proteins levels) with the accumulation of chemical constituents, including their intermediates [108]. The correlation analysis provides clues as to which genes of the plant are involved in the biosynthesis of a particular chemical constituent [109]. Moreover, advanced technologies such as transcriptome sequencing through next generation sequencing technology provides extensive information to facilitate understanding of major change in the metabolic processes

as well as contribute to comparative transcriptomics, evolutionary genomics and gene discovery [110-113].

Picrosides are biosynthesized through a combined route involving mevalonate (MVA), non-mevalonate (MEP), iridoid and phenylpropanoid pathways. Kawoosa et al. [27] cloned two regulatory genes of terpenoid metabolism 3-hydroxy-3-methylglutaryl coenzyme A reductase (pkhmgr) and 1-deoxy-D-xylulose-5-phosphate synthase (pkdxs) from *Picrorhiza*. These genes were cloned to full length followed by cloning of promoter regions to identify cis-acting regulatory elements for light and temperature responsiveness. Electrophoretic mobility shift assay confirmed the binding of proteins to these motifs. The results showed that picrosides biosynthesis is regulated by light and temperature as the expression of two rate limiting enzymes, pkdxs and pkhmgr showed upregulation at low temperature (15°C) and under illumination as compared to 25°C and dark conditions. Picrosides content also showed similar pattern as gene expression. This suggests the importance of light and temperature in regulating pkhmgr and pkdxs, and thus picrosides level in *Picrorhiza*.

In order to gain insight into temperature mediated molecular changes, high throughput de novo transcriptome sequencing and analyses was carried out using plants grown at two temperature conditions 15°C and 25°C using Illumina sequencing technology [32]. Expression profiling in terms of read per exon kilobase per million (RPKM), showed changes in several biological processes and metabolic pathways including cytochrome P450s (CYPs), UDP-glycosyltransferases (UGTs) and those associated with picrosides biosynthesis. Increased picrosides content and upregulation of CYPs and UGTs at 15°C suggested these to be the possible candidates associated with picrosides biosynthesis. RPKM data for all the genes of MEP, MVA pathway and 4-CH, 3-CH, PAL, 4 coumarate CoA liagse, COMT of phenylpropanoid pathway was validated by reverse transcriptase-polymerase chain reaction, wherein expression of majority of the genes was in accordance with the two expression methods. This study identified putative CYPs and UGTs that could help in discovering unknown genes associated with picrosides biosynthesis. However, Picroside-I and Picroside-II contents in tissue samples used for transcriptome analysis differed only by ~1.5 folds.

Further progress was made towards understanding molecular basis of picrosides biosynthesis by Singh et al. [24]. Eight full-length cDNA sequences from MEP and MVA

pathway genes namely, 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXPR), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDS), acetyl-CoA acetyltransferase (ACTH), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), isopentenyl pyrophosphate isomerase (IPPI) and geranyl diphosphate synthase (GDPS) and two partial sequences from phenylpropanoid pathway, PAL and COMT were cloned followed by expression analysis vis-à-vis picrosides content in different tissues. The expression of these genes was highest in leaf tissue followed by rhizome and root which was in accordance with Picroside-I content in these tissues. The younger leaves (first leaf) also exhibited the higher expression of these genes in comparison to older leaves (fourth leaf) which was in correlation with Picroside-I content. The effect of various elicitors like hydrogen peroxide, abscisic acid, methyl jasmonate and salicylic acid was also studied. Picrosides accumulation was enhanced by application of hydrogen peroxide and abscisic acid, whereas methyl jasmonate and salicylic acid treatment decreased the content. Data suggested the importance of genes associated with the synthesis of cinnamate/vanillate moiety in picrosides biosynthesis.

Another endeavour towards understanding the molecular biology of picrosides biosynthesis was made by Pandit et al. [33]. Five genes of MEP and MVA pathway namely ISPD, MECPS, HDS, HMGS and PMK were cloned using comparative genomics and then the expression analysis of all 15 genes of these pathways was done vis-à-vis picrosides content in different tissues and developmental stages of *P. kurroa*. Multiple genes showed elevated expression level of transcripts in different tissues. Four genes of MEP pathway; DXPS, ISPD, ISPE, MECPS and one gene of MVA pathway PMK showed elevated levels of transcripts in shoots and stolons, HDS, DXPR, ISPH and ISPE of MEP pathway showed elevated expression in roots. GDPS was the only gene which showed higher expression in shoots as well as roots. None of the genes showed elevated expression in rhizome tissue which suggested that it only acts as a storage organ for picrosides. The study identified key genes involved in biosynthesis of GPP, the precursor for picrosides biosynthesis.

Identification of few imperative enzymes by differential protein expression using SDS-PAGE followed by MALDI-TOF/TOF MS in *P. kurroa* under picrosides accumulating and non-accumulating conditions was done [114, 115]. However, this study only identified primary metabolic enzymes like NAD(P)H-quinone oxidoreductase, ribulose bisphosphate

carboxylase, fructokinase, glyceraldehyde-3-phosphate dehydrogenase, etc. and one secondary metabolism enzyme, shikimate kinase.

Inspite of several efforts, the complete biosynthetic pathway for picrosides remained elusive. Kumar et al. [28] proposed a plausible biosynthetic pathway for picrosides biosynthesis in *P. kurroa* using a bio-retrosynthetic approach, which includes assembling the biosynthetic pathway from earlier to later steps, i.e. from end-product to its precursor. This can be done by literature survey and fragmentation or dissecting the parent compound. The dissection of parent compound lead to smaller daughter compounds which might be involved in the biosynthesis of the parent complex compound. Fragmentation of compounds must be done according to defined rules such as breaking esterification bond first, removing hydroxylation bonds, double bonds, etc. which generate possible intermediates, which are then searched with their theoretical masses and fragments. Validation of intermediates was done through LC-MS-MS approach which involved developing methods for separation of those intermediates. The intermediates were further confirmed through fragments matching or proposed fragmentation. The presence of different intermediates that were not detected earlier in *P. kurroa* was investigated using LC/ESI–MS/MS method. The presence of catalpol and aucubin, the major backbone structures of picrosides, along with intermediate metabolites boschnaloside, bartsioside and mussaenosidic acid, was confirmed. This study reported complete biosynthetic pathway for picrosides and related compounds in *P. kurroa* for the first time, however identification and validation of genes catalysing corresponding enzymatic steps remained elusive. Bhat et al. [34] identified two uridine diphosphate glycosyltransferases (UGTs), UGT94F4 and UGT86C4 from *P. kurroa*. UGTs are pivotal in the process of glycosylation for decorating natural products with sugars. Full length cDNAs alongwith promoter regions of these UGTs were cloned. UGT94F2 and UGT86C4 showed differential expression pattern in leaves, rhizomes and inflorescence. The presence of cis-regulatory elements within the promoter regions of each gene correlated positively with their expression profiles in response to different phytohormones. Expression profile of UGT94F2 corroborated well with picrosides content which indicated its possible implication in picrosides biosynthesis. Bhat et al. [116] cloned an important rate-limiting gene phenylalanine ammonia lyase (PAL) from phenylpropanoid pathway which supplied precursors like cinnamic acid, vanillic acid, ferulic acid, etc., to a variety of secondary metabolites including picrosides. The expression pattern of PAL was in accordance with

picrosides content in different tissues of *P. kurroa*. The promoter region was also isolated to identify cis-acting regulatory elements.

The precursors for secondary metabolites are provided by primary metabolic pathways such as photosynthesis, glycolysis, pentose phosphate pathway, TCA/Citric acid cycle, etc. Therefore, proper understanding of picrosides biosynthesis requires the coordination of primary and secondary metabolic pathways by tracking events occurring at the molecular level. Recently, a study was carried out [117] to determine the gene expression patterns along with Picroside-I content at different stages of *P. kurroa* growth *viz.* 0, 10, 20, 30 and 40 days of culture *in vitro* in order to identify regulatory steps that provides insight into the metabolic basis controlling the biosynthesis of Picroside-I. It was observed that HK, DXPS, ISPD, HMGR and PMK genes related to primary and secondary metabolic pathways contribute to Picroside-I biosynthesis for the first 20 days of plant growth with the major role of MEP pathway as compared to the MVA pathway. In addition, DAHPS and G-10-H related to shikimate/phenylpropanoid and iridoid pathway, respectively might be responsible for the Picroside-I biosynthesis between 20-30 days of *P. kurroa* growth *in vitro* and thus, hinted as the possible regulators of Picroside-I biosynthesis.

2.8 Molecular characterization of genetic diversity

Genetic diversity can be determined using morphological and molecular markers. Morphological markers are influenced by environmental factors and developmental stage of the plant whereas genetic markers based on DNA sequence are independent of environmental factors and show high levels of polymorphism [39]. DNA markers are reliable for informative polymorphisms as the genetic composition is unique for each species. DNA markers that can correlate DNA fingerprint data with quantity of selected phytochemical markers associated with that particular species have applications in quality control of raw materials [118]. Marker-assisted breeding could be used in developing high secondary metabolite yielding plants. ‘CIM-arogya’, a plant variety has been developed with marker assisted breeding having high artimisinin content in *Artemisia annua* [119].

Majority of the medicinal plant material used by the Ayurvedic and Pharmaceutical industries is collected from the wild/natural sources. Harvesting from the wild has, however, become problematic due to the loss of genetic diversity and habitat destruction. Therefore, there is a need to select proper and appropriate technologies for large-scale production of quality plant material with desired combinations and concentrations of chemical

constituents and also pay attention to the sustainable conservation and utilization of indigenous plant species. *P. kurroa* being an important medicinal herb has been exploited at a very high rate to fulfill the increasing demands of herbal drug industries which has led to the endangered status of this plant, thereby necessitating characterization of genetic diversity for its sustainable conservation. Considerable variation in growth and picrosides content has been observed in different populations of *P. kurroa* but information lacks on genetic characterization of these populations. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in plants and also, the detection of genes influencing economically important traits [21].

Microsatellites or simple sequence repeat (SSR) markers have been widely used for genetic diversity studies due to their advantages over other markers such as multi-allelic, gene specific, codominant, high abundance, high level of polymorphism and high reproducibility. SSR markers have diagnostic and functional significance, and have been usually associated with functional and phenotypic variations. SSRs or microsatellites are short nucleotide repeats of 1-8 bp which vary in length in different individuals. Variation in number of repeats gives the length polymorphism which is identified by amplifying the primers designed for the sequences flanking the SSRs. The number of times the unit is repeated in a given microsatellite can be highly variable, a characteristic that makes them useful as genetic markers. SSRs are generated either de novo from unclassifiable regions in DNA having no such repeats [120] or having variations of already overrepresented simple repeat motifs (minimum requisite of eight repeat units may be essential) followed by mutations [121]. SSRs are highly abundant in diverse plant genomes and have, therefore, become the preferred molecular markers for analysis in plant genetics and ecology. SSRs are more abundant in non-coding region of the genome and generally do not cause any changes in the function of an organism. But, genic SSRs from ESTs or cDNAs being less abundant can have functional significance. SSRs may have some functional roles as zinc finger proteins can bind to many SSR repeats [122]. Although biological roles for SSRs in plants have not been reported as yet, similar roles are expected for these molecular markers in plant genes [123]. Microsatellites or SSRs associated with glucosinolates have been reported in *Arabidopsis thaliana* [124] and lignin, cellulose and hemicellulose in *Eucalyptus globulus* [125]. SSR markers for glucosionolate in *Brassica oleracea* [126] and EST-SSRs linked to genes of carotenoids, tocopherols, folates, glucosinolates, flavonoids and phenylpropanoids pathways in *Brassica rapa* [127] have been identified recently.

Recently, microsatellite sequences were successfully isolated from *P. kurroa* [128]. They are yet to be utilized for the assessment of genetic diversity. Genetic diversity study has been carried out in twenty five accessions of *P. kurroa* using SSR and cytochrome P-450 markers [129]. But, SSR primers from rice microsatellite loci were used in this study. The results showed a high degree of genetic variation among accessions of each eco-geographic region. However, assessment of genetic diversity in *P. kurroa* accessions vis-à-vis picrosides content has not been done so far.

CHAPTER 3

MATERIAL AND METHODS

3.1 Plant Material

Twenty six accessions of *P. kurroa* were obtained from Himalayan Forest Research Institute, Jagatsukh, Manali, Himachal Pradesh (1,900 m altitude, $20^0$35.6′–$32^0$6.1′N and $78^0$57.8′–$77^0$33.7′E) which belonged to different geographical locations and coded as PKS-1 to PKS-26 (Table 3.1). The accessions were grown at Jagatsukh and also planted in the green house controlled environment at the Jaypee University.

Table 3.1 *Picrorhiza kurroa* accessions from different geographical locations used for quantification of picrosides

Accession	Location	District	Altitude	Latitude	Longitude
PKS-1	Hudan	Chamba	3620	33006'27.1"N	76029'171.1"E
PKS-2	Bhuri	Kinnaur	3330	31030'01.3"N	78056'34.5"E
PKS-3	Dhel	Kullu	3597	31045.412″N	77027.680″E
PKS-4	Teita	Chamba	3590	32°31′28″N	76°31′01″E
PKS-5	Moral Danda	Shimla	3354	31018'23.6"N	77045'02.1"E
PKS-6	Malana Jot	Kullu	3144	32°0'19"N	77°14'43"E
PKS-7	Chitkul	Kinnaur	3645	31020'42.2"N	78026'70.2"E
PKS-8	Kundaghat	Kullu	3200	31032.448″N	77024.605″ E
PKS-9	Pulag Nath	Kinnaur	3435	31040'6.2"N	78001'11.9"E
PKS-10	Dalau Pathar	Shimla	2703	31°04′32″N	77°38′36″E
PKS-11	Rohtang	Kullu	3979	32°22′17″N	77°14′47″E
PKS-12	Karguni	Chamba	3064	33003'27.1"N	76026'30.0"E
PKS-13	Gue (Spiti)	Lahaul & Spiti	3671	32°14′46″N	78°02′05″E
PKS-14	Yungpa	Kinnaur	3440	34040'5.1"N	78001'12.0"E
PKS-15	Seri	Chamba	2135	32°14′28″N	76°19′50″E
PKS-16	Salam Tith	Chamba	3440	33059.142″N	77011.173″E
PKS-17	Chander	Kullu	2354	32006431″N	77010.985″E
PKS-18	Sural Bhatori	Chamba	3323	33008'41.6"N	76027'49.4"E
PKS-19	Banjar	Kullu	2866	32010.795″N	77012.974″E
PKS-20	Katgaon	Kinnaur	3115	31039'47.2"N	78000'56.5"E
PKS-21	Pattal (Tissa)	Chamba	3245	32057.321″N	76018.417"E
PKS-22	Tinnu Gaon	Lahaul & Spiti	3238	32°34′49″N	77°07′50″E
PKS-23	Granfu	Lahaul & Spiti	3100	32022'20.0"N	77017'26.4"E
PKS-24	Bhagi	Shimla	3100	31°04′33″N	77°22′22″E

PKS-25	Bhrigu	Kullu	4170	$32^{0}17.556''N$	$77^{0}14.016''E$
PKS-26	Shringul Tung	Shimla	3307	32°14′59″N	78°06′48″E

P. kurroa plants grown at a nursery (Sairopa, 4500m altitude, 31°38' - 31°54'N and 77°20' - 77°45' E) were collected, maintained in greenhouse and cultured in an optimized Murashige and Skoog (MS) medium [130] supplemented with 3 mg/L indole-3-butyric acid and 1 mg/L kinetin in a plant tissue culture chamber maintained at 25±2 ^{0}C with 16 h photoperiod provided by cool white fluorescent light (3,000 lux) [22]. Four *P. kurroa* tissues with varying picrosides content growing in field conditions and tissue culture were taken for gene expression analysis. These included field grown shoots having 2.7% Picroside-I (FGS), tissue cultured shoots having negligible Picroside-I content (TCS), field grown roots having 0.4% Picroside-II content (FGR) and tissue cultured roots with no Picroside-II content (TCR) (Table 3.2).

Table 3.2 Description of *Picrorhiza kurroa* tissues used for RT-qPCR analysis (Pandit et al. [33])

Sample Name	**Description**	**Picroside-I %**	**Picroside-II %**	
FGS	Field grown shoots	2.7	-	
TCS	Tissue cultured shoots grown at 25°C	0.01	-	
FGR	Field grown roots	-	0.4	
TCR	Tissue cultured roots grown at 25°C	-	0.0	

3.2 Market Samples

Dried rhizomes of *Picrorhiza kurroa* were purchased from 3 herbal drug markets in North-India; Delhi, Amritsar and Manali for quality assessment and quantification of picrosides (Table 3.3).

Table 3.3 *Picrorhiza kurroa* rhizomes samples purchased from herbal drug markets of North India for quantification of Picroside-I and Picroside-II

Sample Name	Price in Rs. (per kg)	
Manali (from Manali Market)	450.0	
Himachal Pradesh (from Amritsar Market)	425.0	
Nepal (from Delhi Market)	660.0	
Uttarakhand (from Delhi Market)	250.0	
China (from Delhi Market)	770.0	

3.3 Sample preparation and high performance liquid chromatography (HPLC) analysis

HPLC analysis was done for rhizomes as well as shoots. Dried tissue was ground in a grinder and fresh tissues were ground to a fine powder in liquid nitrogen. 100mg of powdered material was percolated with 10ml 80% methanol overnight at room temperature. The methanolic extract was filtered through 0.22µm membrane filter and 1:10 dilution of filtered extract was made using 80% Methanol. Quantification of picrosides was performed by following the protocol reported by Sood and Chauhan [22] on Waters HPLC System equipped with Waters 515 HPLC pumps, Waters 717 autosampler, Waters 2996 photodiode array detector and Empower software. Waters Spherisorb reverse phase C18 column (4.6mm x 250mm, 5µm) was used and 20µl of sample was injected into the column for analysis. The mobile phase used for the analysis was solvent A (0.05% trifluoro- acetic acid in water) and Solvent B (1:1 methanol/acetonitrile mixture). Solvent A and B were used in

the ratio of 70:30 (v/v). The column was eluted in isocratic mode with a flow rate of 1ml/min at detection wavelength of 270nm. The cycle time of analysis was 30 min at 30°C. The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards (Figure 3.1) procured from ChromaDex, Inc.

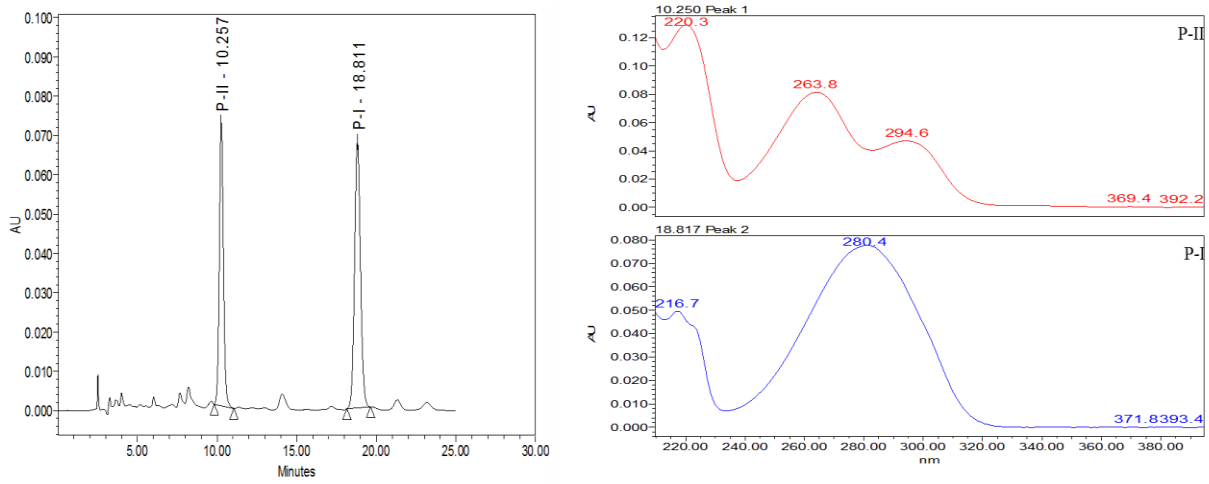
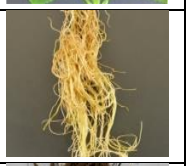


Figure 3.1 Retention time and absorption spectra of Picroside-I and Picroside-II standards

3.4 Generation of *Picrorhiza kurroa* transcriptomes

Six *P. kurroa* tissues with varying contents of Picroside-I and Picroside-II were selected for generation of NGS transcriptomes (Table 3.4). *De novo* whole transcriptome sequencing of *P. kurroa* tissues was done using paired end (PE) sequencing method on Illumina HiSeq 2000 platform. Illumina genome analyzer based sequencing technology (Illumina, USA) yields unique combination of long and short reads, single and paired-end sequencing, strand specificity, and capacity for tens of millions to billions of reads per run with high coverage which allows to annotate coding SNPs, discover novel transcripts, discover transcript isoforms, identify regulatory RNAs, characterize splice junctions, determine the relative abundance of transcripts. For generation of transcriptomes, total RNA was isolated using Purelink miRNA isolation kit (Invitrogen) with minor modifications and RNA quality was checked on 1% denaturing agarose gel by loading 1 µg of total RNA. Approximately 50-60 µg of total RNA for each sample was used for Poly A RNA isolation using micro poly A purist kit (Ambion). The poly A RNA was proceeded for whole transcriptome pair-end library preparation for illumine Hi-Seq 2000.

Table 3.4 Details of *P. kurroa* tissues used for generating NGS transcriptomes

Tissue	**Description**	**Picroside-I (%)**	**Picroside-II (%)**	
PKS-25	Tissue cultured shoots grown at 25°C	0.001	-	
PKS-15	Tissue cultured shoots grown at 15°C	0.6	-	
PKSS	Field grown shoot	2.7	-	
PKR-25	Tissue cultured roots grown at 25°C	-	-	
PKSR	Field grown root	-	0.4	
PKSTS	Field grown stolon	1.77	0.99	

3.4.1 Illumina HiSeq 2000- 2X100 PE library preparation

The pair-end cDNA sequencing libraries were prepared for all samples, separately using illumine TruSeq RNA Library Preparation kit as per protocol. Library preparation was started with mRNA fragmentation followed by reverse transcription, second-strand synthesis, pair-end adapter ligation, and finally ended by amplification of adaptor-ligated library. Library quantification and qualification was performed on Agilent bioanalyzer using High Sensitivity DNA Chip.

3.4.2 Cluster generation and Sequencing run

Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions. The Multiplexing Sample Preparation Oligonucleotide Kit was used to tag libraries for pooling in one flow cell lane. Cluster generation was carried out by

hybridization of template DNA molecules onto the oligonucleotide-coated surface of the flow cell. Immobilized DNA template copies were amplified by bridge amplification to generate clonal DNA clusters. This process of cluster generation was performed on cBOT using TruSeq PE Cluster kit v3-cBot-HS. The kit reagents were used in binding of samples to complementary adapter oligos on paired-end flow cells. The adapters were designed to allow selective cleavage of the forward DNA strand after resynthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment. TruSeq SBS v3-HS kits was used to sequence DNA of each cluster on a flow cell using sequencing by synthesis technology on the HiSeq 2000.

3.4.3 *De novo* assembly

After the quality filtration (mean quality score >=20) and adaptor trimming, the high quality reads for six respective samples were assembled with Velvet/Oases pipeline for different kmer length (kmer 51, 53, 55, 57, 59, 61, 63). From all kmer assemblies, one best kmer assembly was selected for each sample based on N50 and transcriptome length covered and its respective transcript contigs were used for downstream analysis. The assembled transcript contigs were used for GENSCAN gene prediction based on *Arabidopsis* model matrix. Further, CDS obtained for all plant samples were subjected to functional annotation by aligning the transcript contigs to non-redundant database of NCBI using BLASTX program.

3.4.4 Fragment mapping and transcript abundance measurement

Transcript quantification for the assembled transcripts was done using RSEM approach which assesses the transcript abundances based on mapping RNA-Seq reads to the assembled transcriptome. It uses directed graphical model where paired end reads, length of fragment, probability of that read's sequence are modelled as well as lengths of read can vary [131]. RSEM calculates maximum likelihood abundance estimates as well as posterior mean estimates and 95% credibility intervals for genes/isoforms. The number of fragments and fragments per million (FPM) corresponding to each transcript were determined Additionally, FPKM (fragments per kilobase of transcripts per million mapped reads) level measurement was used which is a sensitive approach to detect expression level and measures expression of even poorly expressed transcripts using fragment count.

The methodology followed for de novo whole transcriptome analysis of *P. kurroa* tissues is summarized in Figure 3.2.

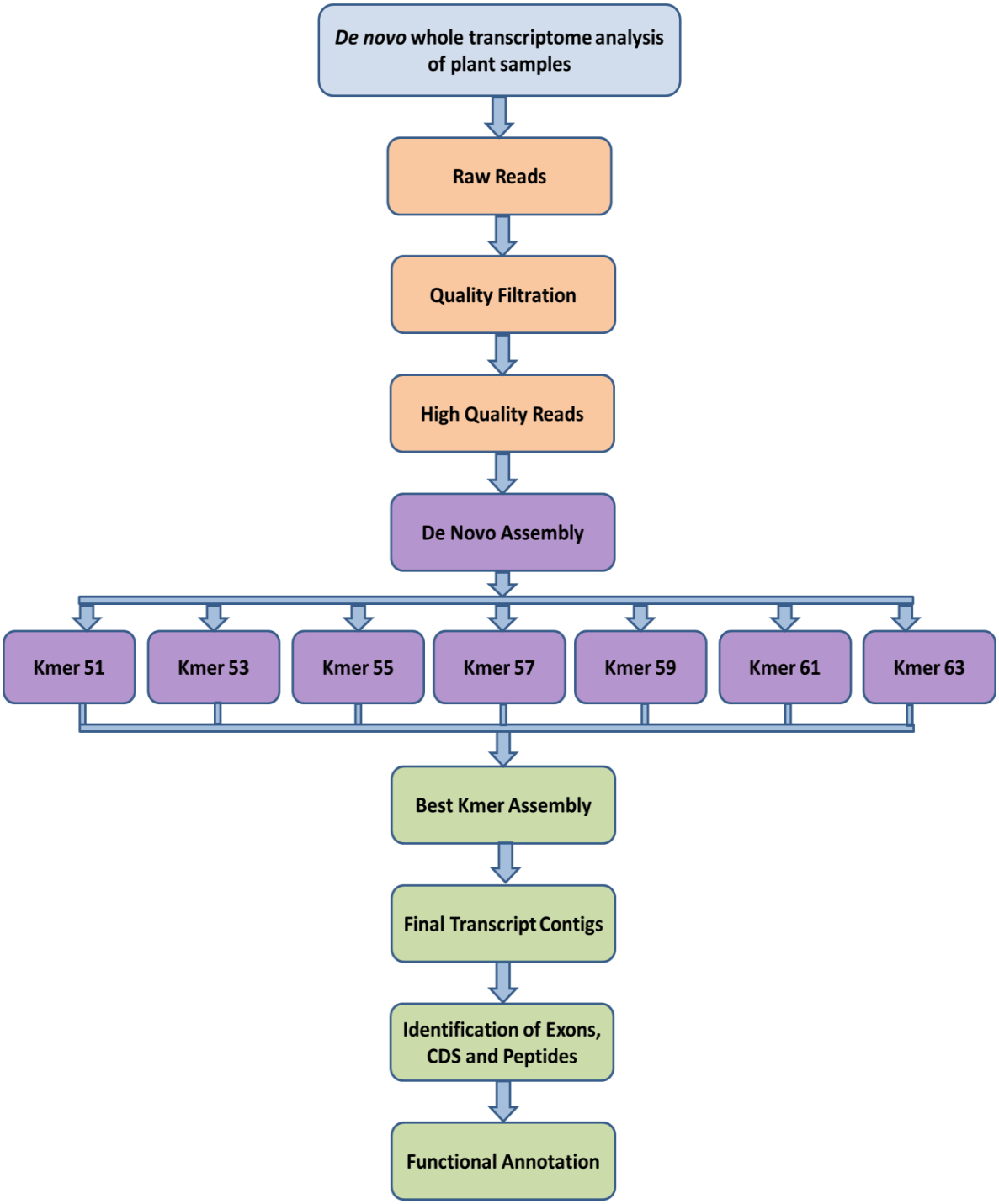


Figure 3.2 Workflow of *de novo* whole transcriptome analysis for *P. kurroa* tissues

3.5 Mapping genes to enzymatic steps of picrosides biosynthetic pathway

The genes catalyzing the enzymatic steps were mapped to complete biosynthetic pathway utilizing information from NGS transcriptomes of *P. kurroa*. The known genes were retrieved from transcriptomes and unknown genes were identified by following the methodology described in Figure 3.3. Initially the enzymes were predicted on the basis of

chemical reactions/group transfer involved in the conversion of one metabolite to the next. All possible enzymes catalysing similar reactions were searched in the KEGG database. The enzymes were then matched in *P. kurroa* transcriptomes using BLAST and shortlisted on the basis of significant similarity. Next, the enzymes were shortlisted based on their functionality i.e. involved in secondary metabolites biosynthesis. The enzymes were then selected on the basis of abundance (FPKM) of corresponding gene transcripts in transcriptomes and finally validated through RT-qPCR in different tissues of *P. kurroa* with varying contents of Picroside-I and Picroside-II. The nucleotide sequences for phenylpropanoid and iridoid pathway genes were extracted from whole genome transcriptomes of *P. kurroa* and gene (paralog) specific primers were designed using online tool Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/).

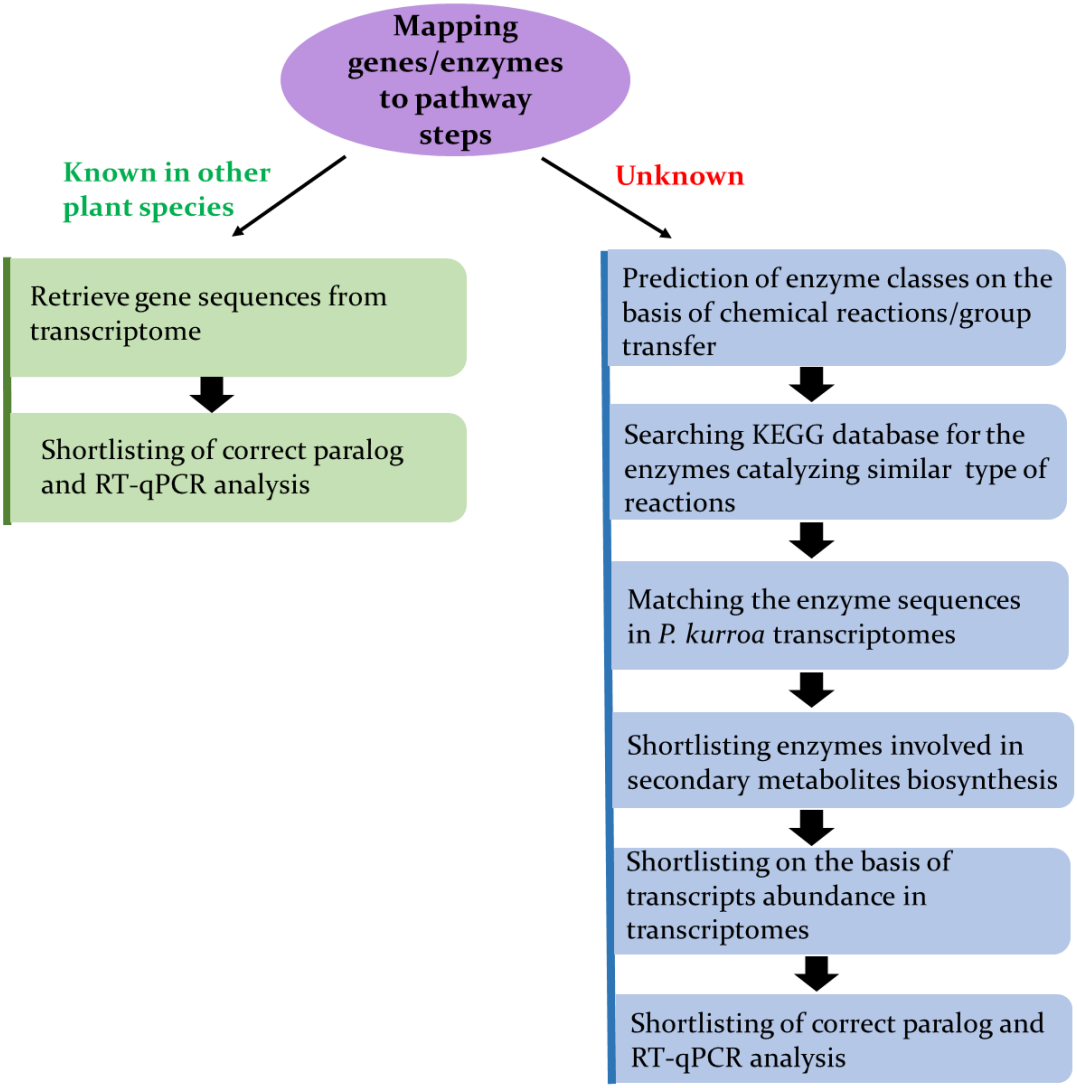


Figure 3.3 Strategy used for mapping genes to enzymatic steps of iridoid biosynthetic pathway

3.6 Identification of appropriate paralogs/transcripts for pathway genes in transcriptomes

Mining of transcriptomes for picrosides biosynthetic pathway genes showed the existence of multiple paralogs of pathway genes (Table 3.5). Therefore, it was required to identify appropriate paralogs/transcripts which was done using the strategy given in Figure 3.4. Three *P. kurroa* transcriptome datasets of tissues PKS-25, PKS-15 and PKSS having progressive increase of 0.001%, 0.6% and 2.7%, respectively in Picroside-I contents were selected for shortlisting correct paralogs. For selection of suitable paralogs of genes, initially multiple sequence alignment of the selected transcripts using ClustalW was carried out to see homology between paralogs for each gene. Further, shortlisting of transcripts was done through BLAST analysis by looking for highest similarity with functionally characterized sequences (retrieved from the NCBI databases which were functionally characterized in the same plant or in other plant species). Thereafter, the transcripts were selected on the basis of transcript abundance (FPKM values), in different transcriptomes generated from *P. kurroa* tissues varying in picrosides contents. The selected paralogs were further validated through RT-qPCR analysis under differential conditions of picrosides biosynthesis.

Table 3.5 Number of paralogs for pathway genes in three shoot transcriptomes (PKS-25, PKS-15 and PKSS) of *Picrorhiza kurroa*

Gene	**No. of paralogs/copies of pathway genes in a transcriptome**		
	PKS-25	**PKS-15**	**PKSS**
ACTH	0	9	8
HMGS	4	5	4
HMGR	6	6	6
MVK	4	2	2
PMK	6	4	2
MVDD	5	3	3
IPPI	6	6	6
GDS	7	8	3
DXPS	17	12	9
DXPR	10	10	7
ISPD	5	2	3
ISPE	4	4	4
MECPS	3	3	2
HDS	1	1	8
ISPH	9	10	10

GS	3	4	3
G10H	0	0	2
10HD	7	12	2
IS	6	7	9
CPM	6	4	2
UGT	58	59	38
ALD	33	35	13
F3D	2	9	13
2HFD	1	1	1
UPD	4	8	5
UGD	11	12	9
SQM	9	3	1
ACT	5	3	0
DAHPS	14	12	10
DQS	5	8	6
CAM	4	6	1
QSD	6	2	2
SK	10	11	5
EPSPS	7	4	4
CS	3	4	8
CM	4	3	2
APD	15	8	9
TAT	1	1	1
PAL	8	7	5
C4H	8	5	5

PKS-25 (Tissue cultured shoots grown at 25°C), PKS-15 (Tissue cultured shoots grown at 15°C), PKSS (Field grown shoot)

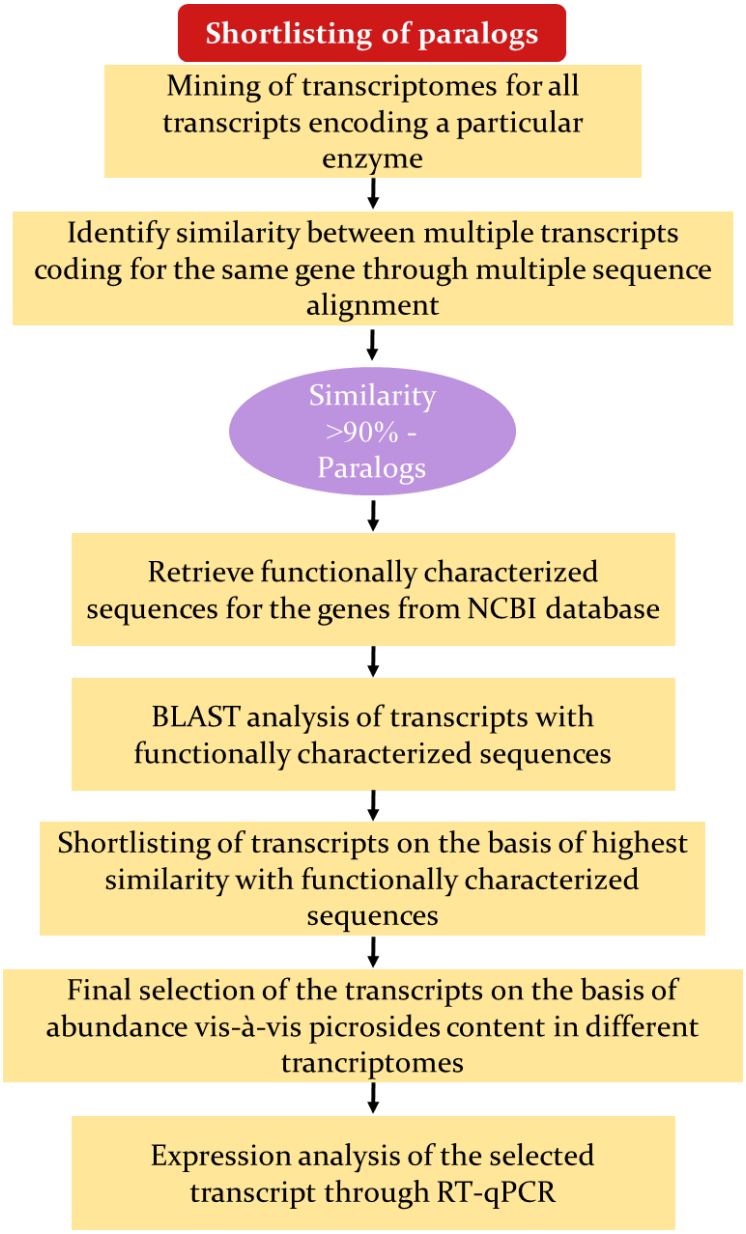


Figure 3.4 Strategy used for shortlisting correct paralogs of Picroside-I and Picroside-II biosynthetic pathway genes

3.7 RNA isolation and cDNA synthesis

Total RNA was isolated from *P. kurroa* tissues by using RaFlex™ total RNA isolation kit (GeNei™) by following manufacturer's instructions. The quality of RNA was checked by 1 % (w/v) ethidium bromide-stained agarose gel. RNA was quantified in a NanoDrop spectrophotometer (Thermo Scientific) by measuring absorbance at 260nm and 280nm and 1µg RNA was used for cDNA synthesis. cDNA was synthesized using Verso cDNA synthesis kit (Thermo Scientific) according to manufacturer's protocol. Concentration of each cDNA sample was adjusted to 100ng/µl for RT-qPCR.

3.8 Expression analysis using reverse transcriptase-quantitative PCR (RT-qPCR)

P. kurroa tissues with varying Picroside-I and Picroside-II contents were selected for correlating the expression status of pathway genes with picrosides content. Equal cDNA quantities (100ng) of each sample were taken. Gene specific primers were checked initially on cDNA for amplification of single product using standard PCR. Quantitative real time PCR was performed using gene specific primers (Table 3.6) in triplicate on a CFX96 system (Bio-Rad Laboratories; Hercules CA) with the iScript one step RT PCR kit (Bio-rad). The PCR protocol was as follows: denaturation for 3 min at 95°C, followed by 39 cycles each of denaturation for 10 s at 95°C, annealing for 30 s at 50–65°C, and elongation for 20 s at 72°C. In final step melt curve analysis was done at 65-95°C with 0.5°C increment at each 0.05 s to verify amplification of a single product. Two housekeeping genes, 26s rRNA and GAPDH were used as internal controls and their average C_t values were taken for normalization. Genes with significantly high expression w.r.t. Picroside-I and Picroside-II content were checked for their expression status in high versus low picrosides content strains (1 each) of *P. kurroa* to validate/ascertain the genes playing significant role in picrosides biosynthesis. The expression status of target genes in response to treatment with their specific inhibitors was also checked on *in vitro* cultured shoots of *P. kurroa* w.r.t. their untreated controls.

Table 3.6 Gene specific primers used for RT-qPCR analysis

Pathway Gene	Forward Primer (FP)	Reverse Primer (RP)	Annealing Temp. (°C)	Expected Amplicon size (bp)
26S	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	58	500
GAPDH	TTGCCATCAATGACCCCTTCA	CGCCCCACTTGATTTTGGA	56	215
ACTH	AGTGTTACTAGAGAGGAGCAGGACA	CCTAGACCTTCATCCTTATCAACAA	50	110
HMGS	GATGGTGCAAGAAAAGGCAACTAGA	GGATATTCACTGGCAAGATTGGGCT	54	110
HMGR	CGTTCATCTACCTTCTAGGGTTCTT	GACATAACAACTTCTTCATCGTCCT	60	100
MVK	ATTAACTCTGAGTATGACGGGTCTG	GAGAGCCCATTTATTTAGCAACTC	50	110
PMK	TGGATGTTGTCGCATCAGCACCTGG	GTAATAGGCAGTCCACTCGCTTCAA	58	100
MVDD	GTAACTCTGGATCCTGACCACCT	TAATACCCCCTCTTTTTCATCCTC	54	100
IPPI	TCTCCTATTCACTGTAAGGGATGTT	ACCACTTAAACAAGAAGTTGTCCAC	54	110
GDS	GATATATGTTCTGAGGGAATGGATG	ATACACCTAGCGAAATTCCTCAACT	55	110
DXPS	ACATTTAAGTTCAAGTCTGGGAGTG	ATGTGCACTCTCTTCTCTTTTAGGA	55.9	110
DXPR	GGAGGAACTATGACTGGTGTTCTT	CAGGTCATAGTGTACGATTTCCTCT	54.9	110
ISPD	GAGAAAAGTGTATCTGTGCTTCTTAG	AATAACCTGCGGTGTATGCATTTCC	56	150
ISPE	TTCATCTAGATAAGAAGGTGCCAAC	CCTCTACCAGTACAATAAGCAGCTC	55	110

MECPS	ATCTATAGCGGCAAACCTACAC	ACTTTAGAGAGGGATGGAGGG	57.1	110
HDS	ATGCCATTTAAGGAACTTGCAACAG	GGAGCACCACCAACATATCCAAATT	58	110
ISPH	CATACTGGGTTGACAGTGATGTAAG	TAAGGACATCTTCAACAGCCTTATC	57.2	110
GS	TGGGTAGATTAGAAGCCAGA	CTGGTGATTTCTACCAGCTC	52	139
G10H	TATCGAGCTTTTCAGTGGAT	GATGTGAGTCCTGTCGATTT	52	136
10HD	GGTAGTGTTTATTGGTGCAG	GATCAACTGATCAAGGTCAA	54	172
IS	AATAAGGCCTTGGTTTATCC	TTAGCCTTAGGATCAACTGC	49	116
MC	AAGGCTGCTCGTACCGATAA	AATGGGGCAGTAGAGTGGTG	58.5	132
CPM	ACCTGAGAGATGGCTAGATG	AGTTCACAGGTTTGTGTTCA	53	188
UGT	AATGGTTGGACTCACAAGAG	ACAAGAAGGTCTGGTTGCTA	55	126
ALD	ACTTTGCTGTGGGATTAAGT	TTCCACAAACTAGAGCAATG	57	174
F3D	AAGAATATGGCTTCTTCCAG	ATCCCTCCAATTATGAACAG	55	197
2HFD	ATTTACTCGACAGGATGTGG	AGCACAAATCAACACCTTCT	52	134
DCH	CATTTCACATCTCCACTGAG	ATGGACAAAGCTTCTTTAGC	53	194
UPD/ UGD	CTGACGGCGTTATTATTTTC/ CATGTTGGACCTTTCAATCT	TCTTCAGAACGGATAGGAGA/ TGGGCCTGAACTCTATCTTA	51/ 51	115/ 109
SQM	GTTGATATACCCGGTCAGAA	TTTCTCAACAGCAGACATGA	51	126
ACT	TATGTCCAAGAATCCAGTGT	AAGAACTCGTCTCGTTCAA	55	111
DAHPS	ACACCATTAAAGCTCCTTGT	TAACAGTCTGAGATCCACCA	59	171
DQS	TGTTCAAAGTGTTGGTGTCT	GTGACATGTGTGGATATGCT	54	192
DQD	AAAAACAACCAGCTTCAAAT	TCTTTGAAACACAATCGAAA	55	193
CAM	GAAGATGCTCCTTCTTATCC	AACACTCGACCAGAATCAC	53	187
QSD	CATAGTTTGGTTCCTCCTCT	ACTGTCTTTCTAGCCCAGAT	52	186
SK	TATGGTGAGAGCTTCTTCAG	AGTTCCGACAGCTGTAATC	56	195
EPSPS	CACAGAACTCAGAAAGTTGG	AGTAATCAGGGAAGGTCTTG	48.5	203
CS	CTCGCTACCAGTCATAAAAG	GAGTGTGTGGTGATTCTGTT	49	185
CM	GTCTACACACCTGCCATTAG	GTACAAATCAGCAACTAGGC	52	198
APD	GCGTATTGGCTATAGAACTG	CGACCTCTGAGAGTAGTTGA	50	192
TAT	CTACATTGAGATCGACTTCC	ACTATCTCCTGAGGACGTTT	53	206
PAL	GCAAGATAGATACGCTCTAA	GTTCCTTGAGACGTCAAT	49	136
C4H	GCAACATTGATGTTCTCAAC	TCCAGCTCTTCAAGGACTAT	53	169

3.9 Enzymes inhibitor treatment vis-a-vis picrosides quantification

Four enzyme inhibitors, Mevinolin (HMGR), Fosmidomycin (DXPR), Glyphosate (EPSPS) and Aminooxyacetic acid-AOA (PAL) inhibiting important enzymatic steps of MEP, MVA and phenylpropanoid pathways were purchased from Sigma-Aldrich, USA (Table 3.7).

Table 3.7 Enzyme inhibitors used for inhibitor assays in *Picrorhiza kurroa* shoot cultures

Inhibitor	Target Enzyme	Pathway module
Mevinolin	HMGR	Mevalonate (MVA)
Fosmidomycin	DXPR	Non-mevalonate (MEP)
Glyphosate	EPSPS	Phenylpropanoid
AOA	PAL	Phenylpropanoid

To prepare stock solutions mevinolin was dissolved in DMSO and rest of the inhibitors were dissolved in autoclaved distilled water and filter sterilized using 0.22 µm sterile filters (Millipore). Shoot apices of length 1 cm were cut from six weeks old cultures of *P. kurroa* shoots raised *in vitro* at 25°C (negligible amount of Picroside-I) and cultured in test tubes containing 10 ml agar gelled MS media containing different concentrations of inhibitors. Inhibitors were added separately at three different concentrations as follows: Mevinolin (2.5 µM, 5 µM, and 10 µM), fosmidomycin (100 µM, 150 µM, and 200 µM), glyphosate (2 mM, 4 mM, 6 mM) and AOA (2 mM, 4 mM, 8 mM). Two controls were also used, one having only agar gelled MS media and hormones another having equivalent concentration of DMSO as used for dissolving mevinolin. After inoculation cultures were incubated at 15±2°C (optimized *in vitro* condition for highest production of Picroside-I (Figure 3.5, [22] Sood and Chauhan, 2010). Shoots were harvested at 15th and 30th day for quantification of picrosides. The experiment was done in triplicates.

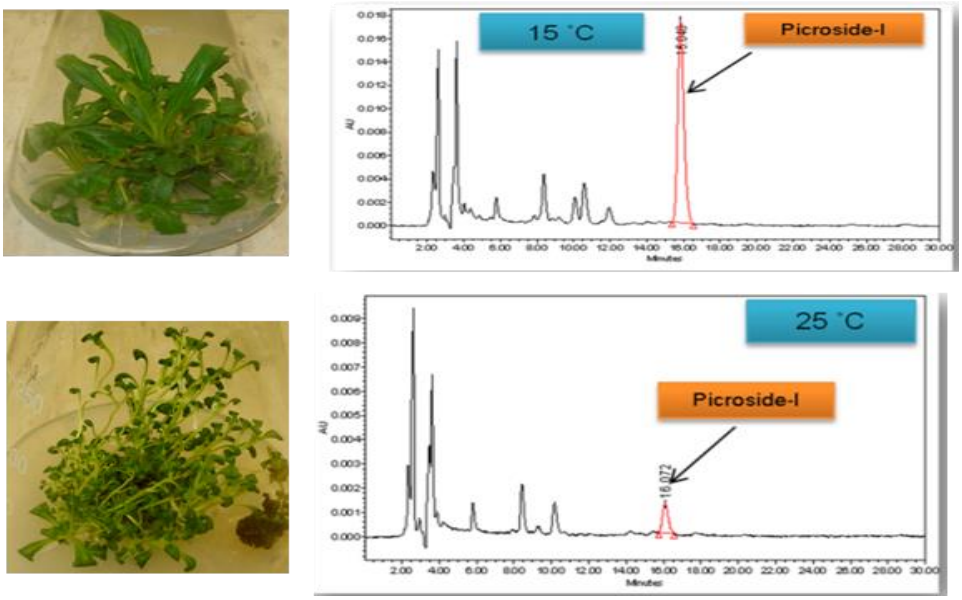


Figure 3.5 HPLC chromatogram for Picroside-I at 15°C and 25°C showing higher amount of Picroside-I at 15°C (Sood and Chauhan [22])

3.10 Identification of SSRs in *P. kurroa* transcriptomes and primer designing

Simple sequence repeats (SSRs) were identified in transcriptomes of *P. kurroa*. An *in-house* designed Perl script was used to locate SSR patterns in the FASTA-formatted sequence files and reported sequence contig ID, SSR motif, number of repeats, and sequence coordinates for each SSR. The FASTA-formatted sequence file was allowed to search for all possible combinations of mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide repeats. The minimum repeat unit was defined as 12 for mono and dinucleotides, 5 for trinucleotides and 3 for all other higher-order motifs, including tetra, penta, and hexanucleotides. Primers were designed from the sequences flanking SSR motifs by using an online primer design tool Primer3 (v. 0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). The target amplicon sizes were set at 100-500 bp with optimal annealing temperature of 52°C and optimal primer length of 20 bp.

3.11 DNA isolation and PCR amplification

Total genomic DNA was isolated from fresh leaves by following the CTAB method developed by Murray and Thompson [132]. The quality of DNA was checked on 0.8% agarose gel and quantified in a NanoDrop spectrophotometer. Concentration of each DNA sample was adjusted to 100ng/µl. PCR reactions were performed in 12.5µl volume and each PCR reaction consisted of 100ng genomic DNA, 0.5µm of each primer, 200µm dNTPs, and 0.5 units *Taq* polymerase. PCR amplification programs included 94°C for 5 minutes, 35 cycles of 94°C for 45 sec, annealing temperature (47–55°C) for 45 sec, 72°C for 2 minutes, and a final extension of 7 minutes at 72°C. 12µl of each PCR product was mixed with 2µl of 6X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in MilliQ water) and subjected to agarose gel electrophoresis using 4% metaphor agarose gel prepared in 1X TAE buffer. The gel was run at a constant voltage of 100 volts for 1.5 to 2 h, stained with ethidium bromide, and analyzed using the gel documentation system AlphaImager EP (Alpha Innotech Corp., USA).

3.12 SSRs Polymorphisms and statistical analysis

Polymorphism among different accessions was assessed by comparing SSR fragments separated according to their respective sizes on agarose gel by comparing with 100bp DNA ladder (Thermo Scientific). A data matrix was generated based on the banding patterns obtained from SSR amplification in twenty six accessions. Population structure of the accessions was assessed using the software program STRUCTURE (version 2.3.3) [133]

with following parameters, length of burnin period and number of MCMC repeats was set to 100000, value of k=1 to 10 with 5 iterations. In our model, admixture was allowed and the allele frequencies were assumed to be independent. This model is recommended as a starting point for most analyses and assumes that individuals may have mixed ancestry which means individual *i* has inherited some fraction of his/her genome from ancestors in population *k*. The output records the posterior mean estimates of these proportions. Pairwise distance matrix was calculated using Dice coefficient. The similarity values were used to generate a dendrogram via the unweighted pair group method with arithmetic mean (UPGMA) using the software DARwin (version 5.0.158) [134] to show a phenetic representation of genetic relationships as revealed by the similarity coefficient. Popgene (version 1.31) [135] was used to measure Nei's gene diversity (h) and Shannon's Information index (I). Cervus 3.0.3 [136] was used to calculate polymorphism information content (PIC), observed heterozygosity (H_{obs}) and expected heterozygosity (H_{exp}).

CHAPTER 4

RESULTS

4.1 Picroside-I and Picroside-II contents in different tissues/organs of *P. kurroa*

Our previous studies have shown that biosynthesis and accumulation of Picroside-I and Picroside-II occurs differentially in different tissues and developmental stages of *P. kurroa* [22, 23, 33]. Picroside-I is produced predominantly in shoots and Picroside-II in roots whereas both accumulate in stolons and rhizomes (Figure 4.1). Differential conditions for the biosynthesis and accumulation of Picroside-I and Picroside-II are not only useful in understanding the biology of their biosynthesis, including biosynthetic pathways but also in regulating the quality of herbal drugs. Conditions with differential contents of Picroside-I and Picroside-II are suitable to associate and ascertain the role and contribution of pathway genes towards their biosynthesis. These studies provided the initial leads for understanding the complete biosynthetic machinery of Picroside-I and Picroside-II in *P. kurroa*.

As picrosides are biosynthesized differentially in *P. kurroa*, therefore it is important to identify an appropriate tissue for quantification of variation in Picroside-I/Picroside-II. The importance of appropriate tissue selection for quantification of picrosides variation is also reflected through our previous study [23]. Picroside-I and Picroside-II were quantified in shoot and rhizome tissues of same genotype of *P. kurroa* of different age groups, first, second and third year. It was observed that shoot tissue contained almost same Picroside-I content in plants of 1-3 years ranging from 0.76±0.5% to 0.8±0.5%. There was negligible difference in Picroside-I content in shoot tissue of different ages of the same genotype. Whereas rhizome tissue showed significant variation for both Picroside-I and Picroside-II in plants of different age groups. Picroside-I content increased from 0.5-0.98% and Picroside-II increased from 0.45-0.81% in 1-3 years. A variation of ~1.4 folds for both Picroside-I and Picroside-II was observed in rhizomes of the same genotype in first, second and third year plants. Rhizome is a storage organ and keep on accumulating picrosides with age. Moreover, different sections of the same rhizome also showed variable amounts of Picroside-I (2.9-8.7%) and Picroside-II (2.2-5.3%), with highest in the uppermost section followed by middle and lower. This shows the limitation of rhizome tissue as uniformity in the plant tissue is necessary to quantify variation in picrosides contents. Therefore,

quantification of Picroside-I in shoots tissue is desirable as it is more informative in assessing the phytochemical variation accurately in *P. kurroa*.

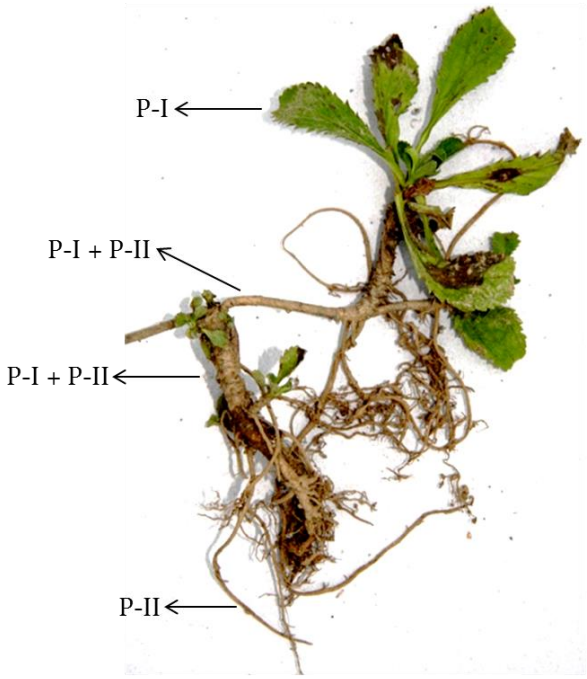


Figure 4.1 Sites of biosynthesis and accumulation of Picroside-I and Picroside-II in *P. kurroa*

4.2 Picrosides content in rhizomes of *Picrorhiza kurroa* from herbal drug markets

P. kurroa rhizome samples purchased from Delhi, Amritsar and Manali markets were from different places namely China, Nepal, Uttarakhand (purchased from Delhi market), Himachal Pradesh (purchased from Amritsar market), Manali (purchased from Manali market). The prices ranged from Rs. 250-550 per kg; though price was much higher (upto Rs. 770 per kg) for plant material imported from China and Nepal.

HPLC analysis showed that the *P. kurroa* plant material from India and Nepal showed higher picrosides content in comparison to plant material from China. The sample from Amritsar market contained the highest picrosides content (10.9%) whereas sample from Chin purchased from Delhi market showed least content (2.8%). Samples from Nepal, Manali and Uttarakhand showed picrosides contents of (7.9%), (8.6) and (6.4%), respectively (Table 4.1). The chromatograms of these samples are shown in Figure 4.2 along with Picroside-I and Picroside-II standards. Therefore, large variation in the total contents of picrosides was observed, which would certainly affect the quality of herbal drug formulations prepared from these raw materials of *P. kurroa*.

Table 4.1 Picroside-I and Picroside-II contents in *P. kurroa* rhizomes purchased from herbal drug markets of North-India

Sample Name	Picroside-I (%)	Picroside-II (%)	Total (%)
Manali (from Manali Market)	3.2	5.4	8.6
Himachal Pradesh (from Amritsar Market)	4.7	6.2	10.9
Nepal (from Delhi Market)	3.3	4.6	7.9
Uttarakhand (from Delhi Market)	2.8	3.6	6.4
China (from Delhi Market)	1.1	1.7	2.8

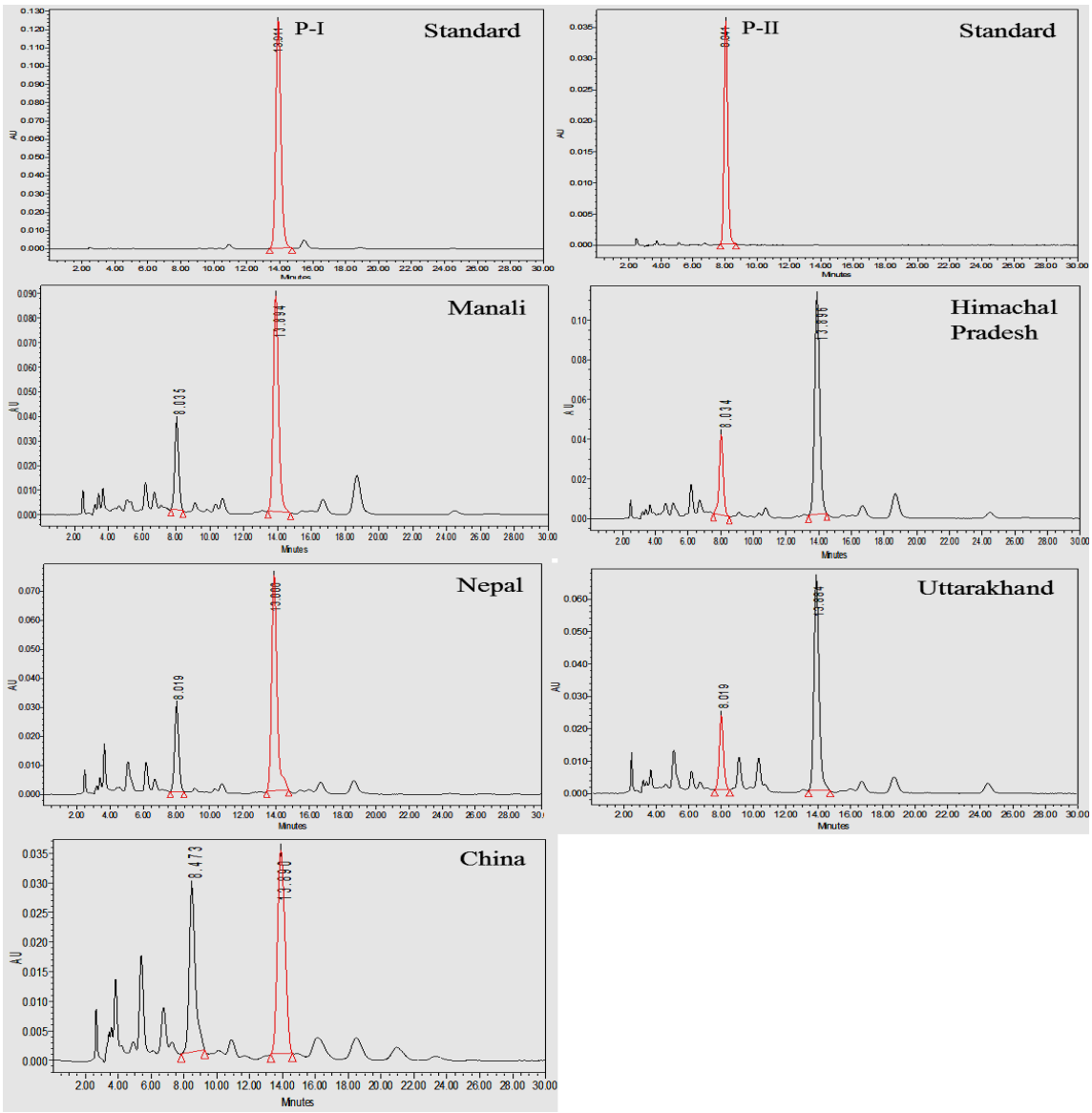


Figure 4.2 HPLC elution profiles of rhizome samples of *P. kurroa* purchased from market along with standard compounds of Picroside-I and Picroside-II

4.3 Identification of chemotypes with higher contents of picrosides

Twenty six accessions of *P. kurroa* were quantified for picrosides content using HPLC. Each sample was taken in duplicates and quantification was repeated four times during the period 2011-2013 to get an accurate estimation of picrosides content. Mature rhizomes (dry) as well as shoots of 26 accessions were quantified for variation in picrosides contents. In rhizomes, Picroside-I content varied from 0.4% to 5.8% whereas Picroside-II content showed variation from 1.4% to 5.4% in twenty six accessions (Figure 4.3). Total picrosides content (Picroside-I+Picroside-II) ranged from 2.9% to 10.9%. The accessions, PKS-1, PKS-2, PKS-3, PKS-5 and PKS-26 showed high amount of Picroside-I ranging from 3.6-5.8% and Picroside-II ranging from 4.6-5.4%. It was observed that rhizomes showed inconsistency in picrosides contents for the same accessions across different years of collection. PKS-1 was the only accession which showed consistently high picrosides content in rhizomes in successive repetitions, therefore it has been identified as a superior genotype. Although other accessions also showed high picrosides content but they were not consistent in successive repetitions. The variable results for the same accessions in different repetitions may be due to the differences in the age of rhizomes at the time of collection. The variation in picrosides content could not be determined appropriately due to erratic picrosides content in rhizomes. Therefore, it was desirable to quantify shoot tissues of these twenty six accessions to assess the accurate variation in picrosides contents.

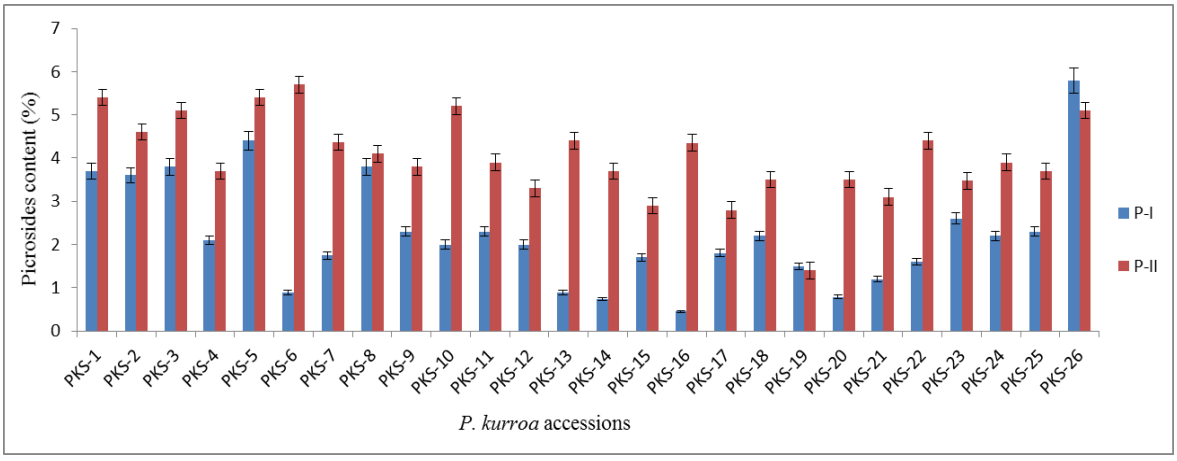


Figure 4.3 Picrosides content variation in rhizomes of *P. kurroa* accessions

Picroside-I content ranged from 0.38% to 2.7% in fresh shoots of 26 accessions in field grown plants and from 0.38% to 2.1% in shoots of greenhouse grown plants (Table 4.2). The highest Picroside-I content (2.7% in field grown shoots and 2.1% in green house shoots)

was shown by accession PKS-1 whereas PKS-4 showed the least Picroside-I content of 0.38%. It was observed that shoot tissue showed consistently similar pattern of Picroside-I content for the same accession in successive repetitions at different time periods across two environmental conditions i.e. field condition and green house condition. There was slight reduction in Picroside-I content in green house shoots as compared to field grown shoots but the accumulation pattern remained consistent. Accessions, PKS-1, PKS-2, PKS-3, PKS-5 and PKS-26 consistently showed high Picroside-I contents from 1.5% to 2.7% in field grown shoots and from 1.5% to 2.1% in green house shoots and have been identified as high Picroside-I content chemotypes. The results showed that a large variation in picrosides content exists in *P. kurroa* populations growing in North-Western Himalayan region.

Table 4.2 Picroside-I contents in field grown and green house grown shoots of different accessions of *P. kurroa*

***P. kurroa* accession code**	**Picroside-I content (%) in field grown shoots (Mean±S.D.)**	**Picroside-I content (%) in green house grown shoots (Mean±S.D.)**
PKS-1	2.70 ± 0.07	2.15 ± 0.24
PKS-2	1.80 ± 0.13	1.54 ± 0.14
PKS-3	1.51 ± 0.20	1.91 ± 0.10
PKS-4	0.38 ± 0.10	0.38 ± 0.07
PKS-5	1.50 ± 0.55	1.71 ± 0.02
PKS-6	0.87 ± 0.41	0.83 ± 0.06
PKS-7	1.14 ± 0.88	1.42 ± 0.06
PKS-8	1.06 ± 0.44	1.04 ± 0.11
PKS-9	0.98 ± 0.32	0.82 ± 0.11
PKS-10	0.95 ± 0.12	0.98 ± 0.15
PKS-11	0.87 ± 0.25	0.88 ± 0.15
PKS-12	0.90 ± 0.45	1.15 ± 0.14
PKS-13	0.81 ± 0.60	1.14 ± 0.05
PKS-14	0.55 ± 0.43	0.77 ± 0.08
PKS-15	0.70 ± 0.42	0.89 ± 0.11
PKS-16	0.55 ± 0.28	0.66 ± 0.14
PKS-17	0.90 ± 0.41	1.09 ± 0.07

PKS-18	0.48 ± 0.21	0.59 ± 0.03
PKS-19	0.92 ± 0.37	0.99 ± 0.03
PKS-20	0.86 ± 0.43	1.13 ± 0.11
PKS-21	0.51 ± 0.08	0.54 ± 0.05
PKS-22	0.88 ± 0.50	1.18 ± 0.03
PKS-23	0.95 ± 0.18	0.87 ± 0.17
PKS-24	0.79 ± 0.24	0.75 ± 0.14
PKS-25	0.76 ± 0.35	0.62 ± 0.02
PKS-26	1.60 ± 0.15	1.66 ± 0.03

4.4 Generation and analysis of NGS transcriptomes of *P. kurroa*

P. kurroa transcriptomes were generated for six tissues having differential contents of Picroside-I and Picroside-II so as to capture all possible genes involved in the biosynthetic machinery of Picroside-I and Picroside-II. Illumina paired-end sequencing generated approximately, 10 million PE reads per sample. The data was generated in FASTQ format. The number of raw reads obtained were 22,705,107 for PKS-25, 25,878,389 for PKS-15, 22,170,403 for PKSS, 26,318,097 for PKR-25, 23,844,570 for PKSR and 21,836,062 for PKSTS. After quality filtering, the highest (24,920,439) and lowest (20,910,870) number of high quality reads were for tissues PKS-15 and PKSTS respectively. In order to select the most appropriate k-mer size for considering *de novo* assembly, bioinformatics softwares Velvet/Oases were run at different k-mer sizes. Best K-mer size was chosen for performing assembly, as it had a balance between over-represented and under-represented transcript numbers, coverage, maximum length obtained and average transcript length. Best Kmer size ranged from 51-57 for six transcriptome datasets.

On the basis of high quality reads 21,056 transcript contigs were assembled with 1,453 bp mean transcript contig length for the tissue PKSS. The maximum and minimum transcript contig length were found to be 30,524 and 509 bp respectively for the tissue PKSS. Sum of the transcript contig lengths was 30,590,943 in bp and the N50 value was 1,532 for PKSS. Exons and CDS were also identified for all six tissue datasets. Assembly statistics for six *P. kurroa* trancriptome datasets is given in Table 4.3. Sequence similarity search was conducted by aligning the transcript contigs to non-redundant (Nr) database of NCBI using BLASTX program for validation and functional annotation of the assembled transcripts.

Table 4.3 Assembly statistics for *P. kurroa* transcriptome datasets

Description	**PKS-25 (Picroside-I 0.001%)**	**PKS-15 (Picroside-I 0.6%)**	**PKSS (Picroside-I 2.7%)**	**PKR-25 (Picroside-II 0%)**	**PKSR (Picroside-II 0.6%)**	**PKSTS (Picroside-I 1.7%, Picroside-II 0.99%)**
Raw reads	22,705,107	25,878,389	22,170,403	26,318,097	23,844,570	21,836,062
High quality reads	21,931,432	24,920,439	21,211,113	23,213,562	22,857,993	20,910,870
Best Kmer	51	51	51	51	55	57
Number of transcript contigs	39,303	37,036	21,056	26,333	20,445	28,310
Sum transcript contigs length in bp	73,560,307	60,632,658	30,590,943	22,897,042	27,671,592	42,852,936
Max transcript contigs length in bp	14,624	16,180	30,524	19000	25,087	18,983
Min transcript contigs length in bp	524	528	509	163	512	519
Mean transcript contigs length in bp	1,871	1,637	1,453	869.51	1,353	1,514
N50 value in bp	2,057	1,761	1,532	950	1,438	1,636
Exons	50,056	46,950	25,202	39,188	22,998	33,431
CDS	39,683	37,355	21,218	26,723	20,499	28,449

PKS-25 (Tissue culture grown shoots at 25°C), PKS-15 (Tissue culture grown shoots at 15°C), PKSS (Field grown shoot), PKR-25 (Tissue culture grown roots at 25°C), PKSR (Field grown root), PKSTS (Field grown stolon)

4.5 Mapping genes to enzymatic steps in Picroside-I and Picroside-II biosynthetic pathways

Complete biosynthetic pathway for Picroside-I and Picroside-II involving different integrating modules, MVA, MEP, iridoid and phenylpropanoid was deciphered in our laboratory [28] but genes catalyzing the corresponding enzymatic steps were not identified. The enzymatic steps of MVA and MEP were known in *P. kurroa* whereas only partial information was available for the enzymatic steps of phenylpropanoid and iridoid pathway. The genes corresponding to enzymatic steps of phenylpropanoid pathways were mapped directly from transcriptome as these were known in other plants but not in *P. kurroa*. 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 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. To identify the unknown genes, we designed an approach involving transcriptomics and KEGG database information as described in Figure 3.3 in methods section. The eighth step in the pathway is conversion of boschnaloside to 8-epideoxy loganic acid which involves hydroxylation/oxidation reaction. The similar type of reaction is catalyzed by aldehyde dehydrogenase (ALD) and thus we proposed that ALD might also catalyze this reaction. The ninth step is conversion of 8-epideoxy loganic acid to mussaenosidic acid, which is catalysed by a hydroxylase enzyme. We proposed flavanone 3-dioxygenase/hydoxylase (F3D) for this reaction as this enzyme has been known to catalyze similar type of reaction. The tenth step is conversion of mussaenosidic acid to deoxygeniposidic acid, which is catalyzed by dehydratase enzyme. We searched for all the dehydratases present in *P. kurroa* transcriptomes and looked for their involvement in secondary metabolism. 2-hydroxyisoflavanone dehydratase (2HFD) was the only dehydratase involved in secondary metabolites biosynthesis, therefore, it was predicted to be catalysing a similar reaction in *P. kurroa*. The eleventh step is conversion of deoxygeniposidic acid to geniposidic acid, which involves hydroxylation reaction. The similar reaction is catalyzed by deacetoxycephalosporin-C hydroxylase and thus we predicted that this enzyme might also catalyze the eleventh step reaction in *P. kurroa*. The twelth step is conversion of geniposidic acid to bartsioside, which is catalyzed by a decarboxylase enzyme. Two decarboxylases, uroporphyrinogen decarboxylase (UPD) and udp-glucuronic acid decarboxylase (UGD) were identified from *P. kurroa* transcriptomes. Uroporphyrinogen decarboxylase catalyzes the decarboxylation of uroporphyrinogen to

coproporphyrinogen. Similar chemical reaction is involved in the conversion of geniposidic acid to bartsioside. The thirteenth step is the conversion of bartsioside to aucubin and this reaction is similar to ninth step, which is being catalyzed by a similar enzyme flavanone 3-dioxygenase/hydroxylase (F3D). Fourteenth step is conversion of aucubin to catalpol which is catalyzed by an epoxidase or monooxygenase enzyme. We searched for all the monooxygenases and epoxidases in *P. kurroa* transcriptomes and identified two enzymes squalene monooxygenase (SQM) and squalene epoxidase (SQE) involved in secondary metabolism but squalene epoxidase did not show significant expression w.r.t. Picroside-I or Picroside-II contents. The last step in the formation of Picroside-I and Picroside-II from catalpol involves acyl group transfer reaction and we predicted this reaction to be catalyzed by anthocyanin acyltransferase (ACT). The complete biosynthetic pathway with the identification of all genes encoding corresponding enzymatic steps is shown in Figure 4.4.

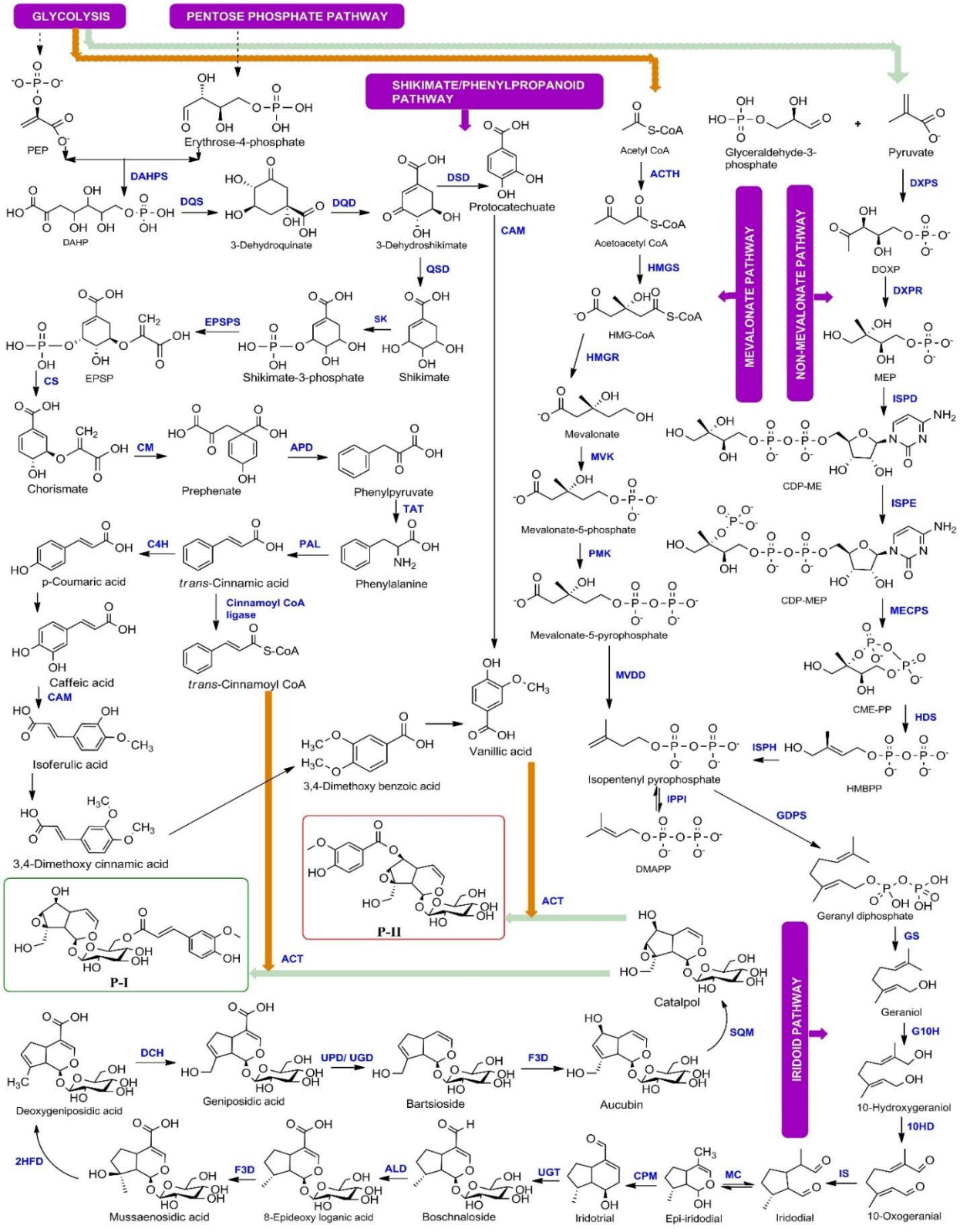


Figure 4.4 Complete biosynthetic pathway for Picroside-I and Picroside-II of *Picrorhiza kurroa* with enzymatic steps. The metabolites have previously been identified by Kumar et al. [28]

4.6 Identification of appropriate paralogs/isoforms for picrosides biosynthetic pathway genes

The previous studies on expression analysis of picrosides biosynthetic pathway genes have not taken into consideration the importance of correct paralog selection for expression profiling vis-à-vis picrosides contents [24, 27, 34, 116]. But there can be multiple paralogs of genes involved in picrosides biosynthesis, each paralog with different transcript abundance and it is not necessary that all the paralogs are carrying out the similar function. Therefore it is important to identify a correct paralog to determine the expression level of a gene accurately. Mining of *P. kurroa* transcriptomes for picrosides biosynthetic pathway genes showed the existence of multiple paralogs for each pathway gene. Three transcriptome datasets of tissues PKS-25, PKS-15 and PKSS with progressive increase in Picroside-I contents were selected for shortlisting correct paralogs. The appropriate paralog for each gene was identified and selected for expression analysis using the methodology described in methods section (Figure 3.4). The correct paralog for each pathway gene was selected to validate its expression level in different tissues of *P. kurroa*. The paralogs selected for pathway genes in different shoot transcriptomes of *P. kurroa* are given in Table 4.4.

Table 4.4 Paralogs selected for Picroside-I and Picroside-II biosynthetic pathway genes from *P. kurroa* transcriptomes

Genes	Functionally characterized NCBI Accession	Highest blast similarity with NCBI accession			Transcript abundance (FPKM)			Selected transcript ID	Highest similarity (with PKSS) of PKS-15 and PKS-25 transcript	
		Transcript ID (%Query coverage, %Similarity) (Size in bp)								
		PKS-25	**PKS-15**	**PKSS**	**PKS-25**	**PKS-15**	**PKSS**		**PKS-15**	**PKS-25**
ACTH	*C. roseus* JF739870.1 (1266 bp)	-	PKS-15_14759 (95, 82) (1215 bp)	PKSS_5199 (95, 82) (1215 bp)	-	6.3	25.5	PKSS_5199	100, 99	-
HMGS	*C. sinensis* JQ390224.1 (1829 bp)	PKS-25_16760 (75, 79) (1383 bp)	PKS-15_12688 (75, 79) (1383 bp)	PKSS_4343 (75, 79) (1383 bp)	34.2	20	133.2	PKSS_4343	100, 99	100, 99
HMGR	*E. pekinensis* EF062569.1 (2200 bp)	PKS-25_8607 (67, 76) (1701 bp)	PKS-15_3158 (66, 76) (1659 bp)	PKSS_2765 (66, 75) (1695 bp)	23	47.2	47.9	PKSS_2765	87, 75	83, 76
MVK	*B. monnieri* JQ670899.1 (1307 bp)	PKS-25_22471 (88, 80) (1164 bp)	PKS-15_15808 (88, 80) (1164 bp)	PKSS_5807 (88, 80) (1164 bp)	8.4	19.5	19.2	PKSS_5807	100, 99	100, 99
PMK	*C. roseus* HM462020.1 (1555 bp)	PKS-25_14643 (95, 75) (1521 bp)	PKS-15_27190 (54, 74) (888 bp)	PKSS_5563 (90, 75) (1419 bp)	22.4	34.4	30.3	PKSS_5563	57, 99	100, 99
MVDD	*P. ginseng* GQ455989.2 (1254 bp)	PKS-25_19258 (99, 78) (1260 bp)	PKS-15_17630 (98, 78) (1239 bp)	PKSS_7386 (99, 780) (1260 bp)	19.6	18.7	33.9	PKSS_7386	98, 99	100, 99
IPPI	*I. batatas* DQ150100.1 (1155 bp)	PKS-25_25624 (61, 81) (708 bp)	PKS-15_23153 (61, 80) (708 bp)	PKSS_14086 (61, 80) (921 bp)	96.9	38.8	108.2	PKSS_14086	100, 99	100, 99
GDS	*V. vinifera* AY351862.1 (1028 bp)	PKS-25_19858 (96, 77) (1260 bp)	PKS-15_19184 (96, 77) (1241 bp)	PKSS_17117 (64, 77) (672 bp)	12.4	12.8	26.3	PKS-15_19184	100, 94	100, 96
DXPS	*S. rebaudiana* AJ429232.2 (2142 bp)	PKS-25_6679 (86, 71) (2184 bp)	PKS-15_3519 (86, 71) (2190 bp)	PKSS_3192 (67, 73) (1461 bp)	19.7	25.1	0	PKS-15_3519	99, 84	99, 85
DXPR	*C. acuminata* DQ355159.1 (1823 bp)	PKS-25_12386 (74, 75) (1428 bp)	PKS-15_8628 (74, 75) (1428 bp)	PKSS_3705 (74, 75) (1428 bp)	126.8	23.5	34.8	PKSS_3705	100, 99	100, 99
ISPD	*G. biloba* DQ102360.1 (1411 bp)	PKS-25_33913 (49, 74) (720 bp)	PKS-15_23679 (49, 74) (720 bp)	PKSS_16533 (49, 74) (717 bp)	9.3	53.9	18.2	PKSS_16533	99, 94	99, 94

ISPE	*G. biloba* DQ102358.1 (1800)	PKS-25_17580 (44, 73) (1221 bp)	PKS-15_9878 (44, 73) (1221 bp)	PKSS_6777 (44, 74) (1221 bp)	39.3	32.8	16	PKSS_6777	100, 99	100, 99
MECPS	*G. biloba* AY971576.1 (836 bp)	PKS-25_33833 (57, 77) (684 bp)	PKS-15_33598 (57, 77) (684 bp)	PKSS_18307 (57, 77) (684 bp)	74.7	45.8	37.1	PKSS_18307	100, 99	100, 99
HDS	*S. rebaudiana* DQ768749.4 (2599 bp)	PKS-25_6066 (85, 79) (2229 bp)	PKS-15_3447 (85, 79) (2229 bp)	PKSS_2512 (65, 81) (1701 bp)	85	51.8	34.7	PKS-15_3447	100, 89	100, 89
ISPH	*P. kurroa* EF199770.1 (1713 bp)	PKS-25_17704 (81, 93) (1380 bp)	PKS-15_12216 (81, 98) (1389 bp)	PKSS_4591 (81, 97) (1389 bp)	147.5	39.6	21.2	PKSS_4591	100, 99	100, 92
GS	*C. roseus* JN882024.1 (1770 bp)	PKS-25_14891 (71, 73)* (1770 bp)	PKS-15_10718 (72, 74)* (1629 bp)	-	29.8	27.7	-	PKS-15_10718	100, 83*	
G10H	*C. roseus* KF561461.1 (1482 bp)	-	-	PKSS_14268 (50, 89) (861 bp)	-	-	18.9	PKSS_14268	-	-
10HD	*C. roseus* KF302069.1 (1137 bp)	PKS-25_16819 (97, 75) (1128 bp)	PKS-15_21110 (97, 74) (1128 bp)	PKSS_13246 (75, 74) (858 bp)	170.4	93.3	19.3	PKS-15_21110	100, 90	100, 99
IS	*C. roseus* JX974564.1 (1167 bp)	PKS-25_20798 (93, 66) (1179 bp)	PKS-15_20867 (93, 66) (1197 bp)	PKSS_10371 (93, 66) (1131 bp)	82.6	14.9	68.7	PKSS_10371	99, 99	99, 99
CPM	*M. truncatula* DQ335800.1 (1719 bp)	PKS-25_16069 (81, 86) (1509 bp)	PKS-15_11131 (81, 68) (1509 bp)	PKSS_3827 (81, 68) (1509 bp)	69	70	112.2	PKSS_3827	100, 99	100, 99
UGT	*P. kurroa* JQ996408.1 (1422 bp)	PKS-25_22805 (84, 99) (1428 bp)	PKS-15_13757 (100, 98) (1428 bp)	PKSS_3392 (99, 98) (1428 bp)	61.9	19.7	73.4	PKSS_3392	99, 99	98, 71
ALD	*A. thaliana* AY056398.1 (1709 bp)	-	-	PKSS_4439 (85, 70) (1515 bp)	-	-	52.4	PKSS_4439	-	-
F3D	*G. max* FJ770474.1 (1134 bp)	PKS-25_23477 (85, 38) (1011 bp)	PKS-15_22234 (85, 37) (1011 bp)	PKSS_5831 (88, 37) (1011 bp)	11.4	12	186.4	PKSS_5831	100, 89	100, 96
2HFD	*G. max* AB154415.1 (960 bp)	PKS-25_30081 (46, 69) (909 bp)	PKS-15_23748 (46, 69) (909 bp)	PKSS_8401 (46, 69) (969 bp)	10.1	15.5	35	PKSS_8401	93, 99	93, 99
UPD	*M. truncatula* XM_003600989.1 (1398 bp)	PKS-25_22449 (72, 77) (1209 bp)	PKS-15_18082 (72, 77) (1314 bp)	PKSS_6747 (72, 77) (1155 bp)	113.9	0.2	12.7	PKSS_6747	95, 90	95, 90

UGD	*G. hirsutum* EU817581.1 (1311 bp)	PKS-25_17015 (64, 76) (1296 bp)	PKS-15_15606 (76, 82) (1317 bp)	PKSS_6772 (77, 82) (1197 bp)	20.3	21.7	24.2	PKSS_6772	100, 99	100, 99
SQM	*T. cacao* XM_007048297.1 (1874 bp)	PKS-25_11305 (90, 79) (1590 bp)	PKS-15_9913 (74, 75) (1590 bp)	PKSS_10700 (67, 84) (1077 bp)	43.9	30	41.8	PKSS_10700	100, 99	100, 99
ACT	*E. sagittatum* KJ010528.1 (1564 bp)	PKS-25_15966 (99, 35)* (1404 bp)	PKS-15_12041 (99, 35)* (1404 bp)	-	31.5	51.2	-	PKS-15_12041	100, 100*	
DAHPS	*N. tabacum* M64261.1 (1928 bp)	PKS-25_18536 (73, 81) (1506 bp)	PKS-15_12601 (73, 81) (1599 bp)	PKSS_3016 (73, 81) (1599 bp)	30.4	31.6	152.8	PKSS_3016	100, 98	92, 86
DQS	*V. vinifera* FJ604857.1 (1371 bp)	PKS-25_20294 (80, 79) (1359 bp)	PKS-15_15388 (80, 79) (1359 bp)	PKSS_4974 (80, 79) (1359 bp)	34.9	15.4	31.3	PKSS_4974	100, 99	100, 98
CAM	*V. planifolia* AY555144.1 (1219 bp)	PKS-25_ 26225 (43, 70) (1050 bp)	PKS-15_ 22575 (43, 67) (1113 bp)	PKSS_16915 (43, 70) (858 bp)	14.4	6.6	12.2	PKSS_16915	84, 74	100, 97
QSD	*M. truncatula* XM_003608150.1 (1521 bp)	PKS-25_12191 (98, 66) (1545 bp)	PKS-15_9901 (83, 71) (1545 bp)	PKSS_8245 (78, 71) (1245 bp)	9.9	22.1	25.1	PKSS_8245	100, 99	100, 99
SK	*O. sativa* AB188834.1 (927 bp)	PKS-25_35453 (64, 67) (663 bp)	PKS-15_24906 (64, 66) (873 bp)	PKSS_11709 (64, 67) (873 bp)	27.8	13.1	44.3	PKSS_11709	99, 99	75, 99
EPSPS	*Z. japonica* GU256772.1 (1268 bp)	PKS-25_14110 (95, 74) (1557 bp)	PKS-15_11760 (98, 73) (1536 bp)	PKSS_6421 (88, 74) (1158 bp)	39.7	23.3	54	PKSS_6421	99, 92	100, 99
CS	*V. vinifera* FJ604855.1 (1312 bp)	PKS-25_17043 (87, 81) (1326 bp)	PKS-15_12629 (87, 81) (1326 bp)	PKSS_4687 (87, 81) (1320 bp)	62.5	35.8	42.1	PKSS_4687	99, 97	99, 97
CM	*A. thaliana* L47355.1 (1006 bp)	PKS-25_26162 (58,66) (963 bp)	PKS-15_29160 (53, 66) (933 bp)	PKSS_11804 (58, 66) (963 bp)	70.8	23.6	26.1	PKSS_11804	96, 99	100, 99
APD	*M. truncatula* XM_003610396.1 (1443 bp)	PKS-25_21892 (64, 76) (1182 bp)	PKS-15_17733 (65, 74) (1182 bp)	PKSS_7746 (62, 76) (1020 bp)	6.4	5.7	18.3	PKSS_7746	100, 95	100, 99
TAT	*G. max* DQ003328.1 (1709 bp)	PKS-25_27461 (43, 74)* (804 bp)	PKS-15_27179 (43, 74)* (804 bp)	-	15	13.5	-	PKS-15_27179	99, 100*	

PAL	*P. kurroa* JQ996410.1 (2312 bp)	PKS-25_7770 (91, 82) (2133 bp)	PKS-15_14278 (55, 84) (1296 bp)	PKSS_1122 (91, 82) (2133 bp)	11.3	18.8	78.1	PKSS_1122	60, 99	100, 98
C4H	*C. sinensis* AY641731.2 (1352 bp)	PKS-25_10021 (86, 79) (1947 bp)	PKS-15_15422 (86, 79) (1518 bp)	PKSS_7335 (86, 79) (1341 bp)	167.9	246.6	193.5	PKSS_7335	99, 99	99, 99

PKS-25 (Tissue culture grown shoots at 25°C), PKS-15 (Tissue culture grown shoots at 15°C), PKSS (Field grown shoot), FPKM (Fragments per kilobase of transcripts per million mapped reads)

4.7 Expression analysis of Picroside-I and Picroside-II biosynthetic pathway genes vis-à-vis picrosides content

The results are in continuation to our previous work which reported the expression status of 15 genes of MEP and MVA pathway in different tissues of *P. kurroa* [33]. Expression analysis of remaining genes of the biosynthetic pathway (15 genes from iridoid pathway and 12 from phenylpropanoid pathway) was done in four tissues varying for Picroside-I and Picroside-II contents. Majority of the genes showed higher expression in relation to picrosides content. Eleven genes of MEP and MVA pathways (HMGS, HMGR, MVK, PMK, MVDD, GDPS, DXPS, ISPD, ISPE, MECPS and ISPH) showed ~11-166 folds higher expression in field grown shoots (FGS) having 2.7% Picroside-I content w.r.t. tissue cultured shoots (TCS) having negligible (0.01%) Picroside-I whereas only one gene of MEP pathway showed elevated transcript abundance of 12 folds in field grown roots (FGR) having 0.4% Picroside-II content wr.t. tissue cultured roots (TCR) having no Picroside-II content. Four genes of iridoid pathway (GS, 2HFD, DCH and SQM) and two genes of phenylpropanoid pathway (DQS and TAT) showed elevated levels of transcripts (~12-130 folds) in FGS w.r.t. TCS. Two genes of iridoid pathway (F3D and ACT) and six genes of phenylpropanoid pathway (DQD, QSD, CAM, SK, EPSPS and PAL) showed ~16-96 folds expression in FGR wr.t. TCR. Four genes of iridoid pathway (G10H, CPM, ALD and UPD/UGD) and two genes of phenylpropanoid pathway (CM and APD) showed ~12-42 folds higher expression in FGS as well as FGR w.r.t. their tissue cultured counterparts. The difference in fold expression in field grown tissues in comparison to tissue culture grown tissues for MEP, MVA, iridoid and phenylpropanoid pathways is given in Figure 4.5 (A, B, C, D, E and F). The pathway genes were categorized into three classes on the basis of folds expression for Picroside-I and Picroside-II (Table 4.5). The modular architecture of picrosides biosynthetic pathway depicting the expression status of genes for Picroside-I and Picroside-II is given in Figure 4.6.

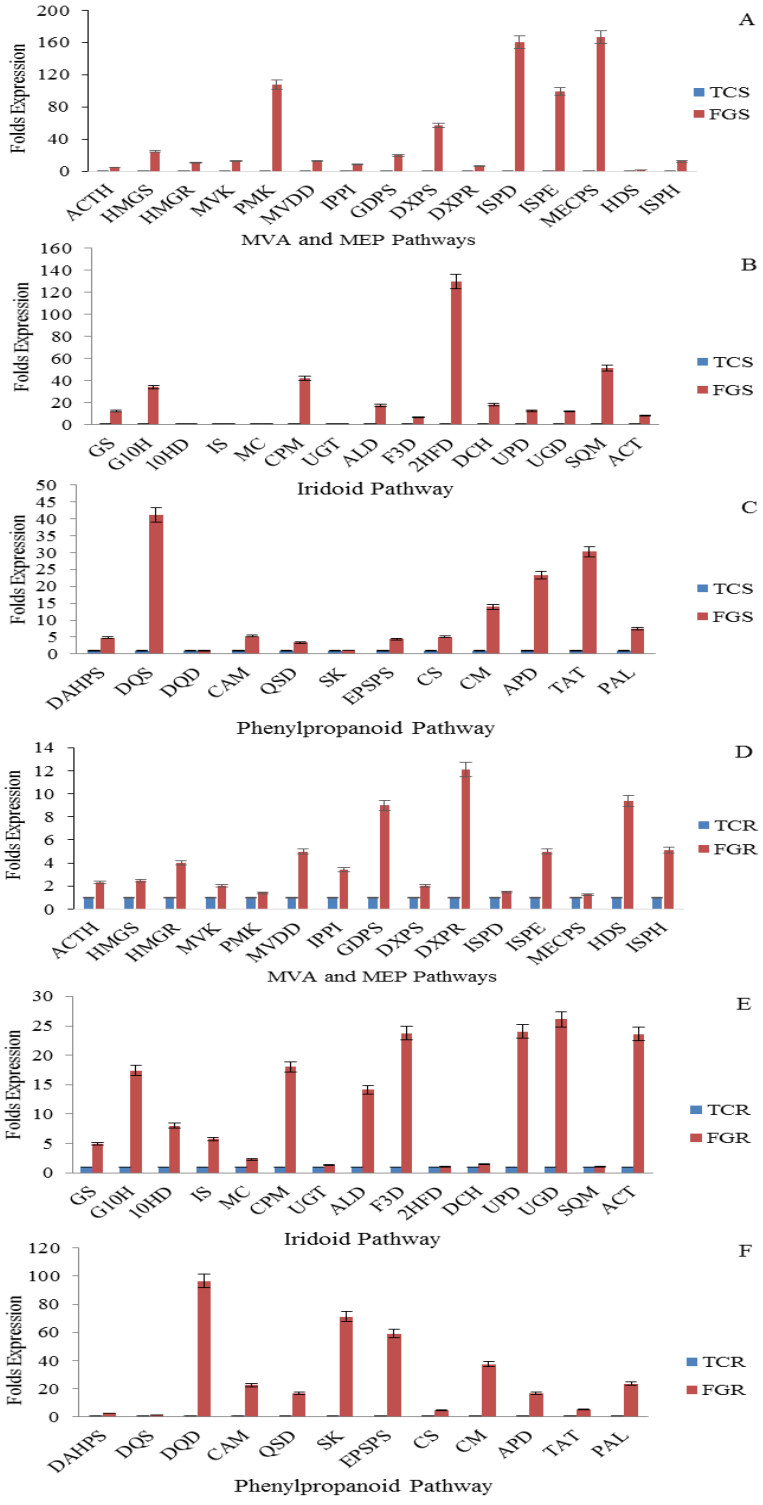


Figure 4.5 Expression status of MVA, MEP, iridoid and phenylpropanoid pathway genes in field grown shoots (FGS) having 2.7% Picroside-I w.r.t. tissue cultured shoots (TCS) having 0.01% Picroside-I (A, B, C) and in field grown roots (FGR) having 0.4 % Picroside-II w.r.t. tissue cultured roots (TCR) having 0.0% Picroside-II (D, E, F). MVA and MEP pathway genes data from Pandit et al. [33]

Table 4.5 Classification of Picroside-I and Picroside-II biosynthetic pathway genes based on fold increase in transcript abundance

Pathway/ Genes	**Picroside-I**			**Picroside-II**		
	1-20 folds	**21-40 folds**	**Above 40 folds**	**1-20 folds**	**21-40 folds**	**Above 40 folds**
MVA	ACTH, HMGR, MVK, MVDD, IPPI, GDS	HMGS	PMK	ACTH, HMGS, HMGR, MVK, PMK, MVDD, IPPI, GDS	-	-
MEP	DXPR, HDS, ISPH	-	DXPS, ISPD, ISPE, MECPS	DXPS, DXPR, ISPD, ISPE, MECPS, HDS, ISPH	-	-
Iridoid	GS, 10HD, IS, MC, UGT, ALD, F3D, DCH, UPD, UGD, ACT	G10H	CPM, 2HFD, SQM	GS, G10H, 10HD, IS, MC, CPM, UGT, ALD, 2HFD, DCH, SQM	F3D, UPD, UGD, ACT	-
Phenylpropanoid	DAHPS, DQD, QSD, SK, EPSPS, CS, CM, PAL	APD, TAT	DQS, CAM	DAHPS, DQS, QSD, CS, APD, TAT	CAM, CM, PAL	DQD, SK, EPSPS

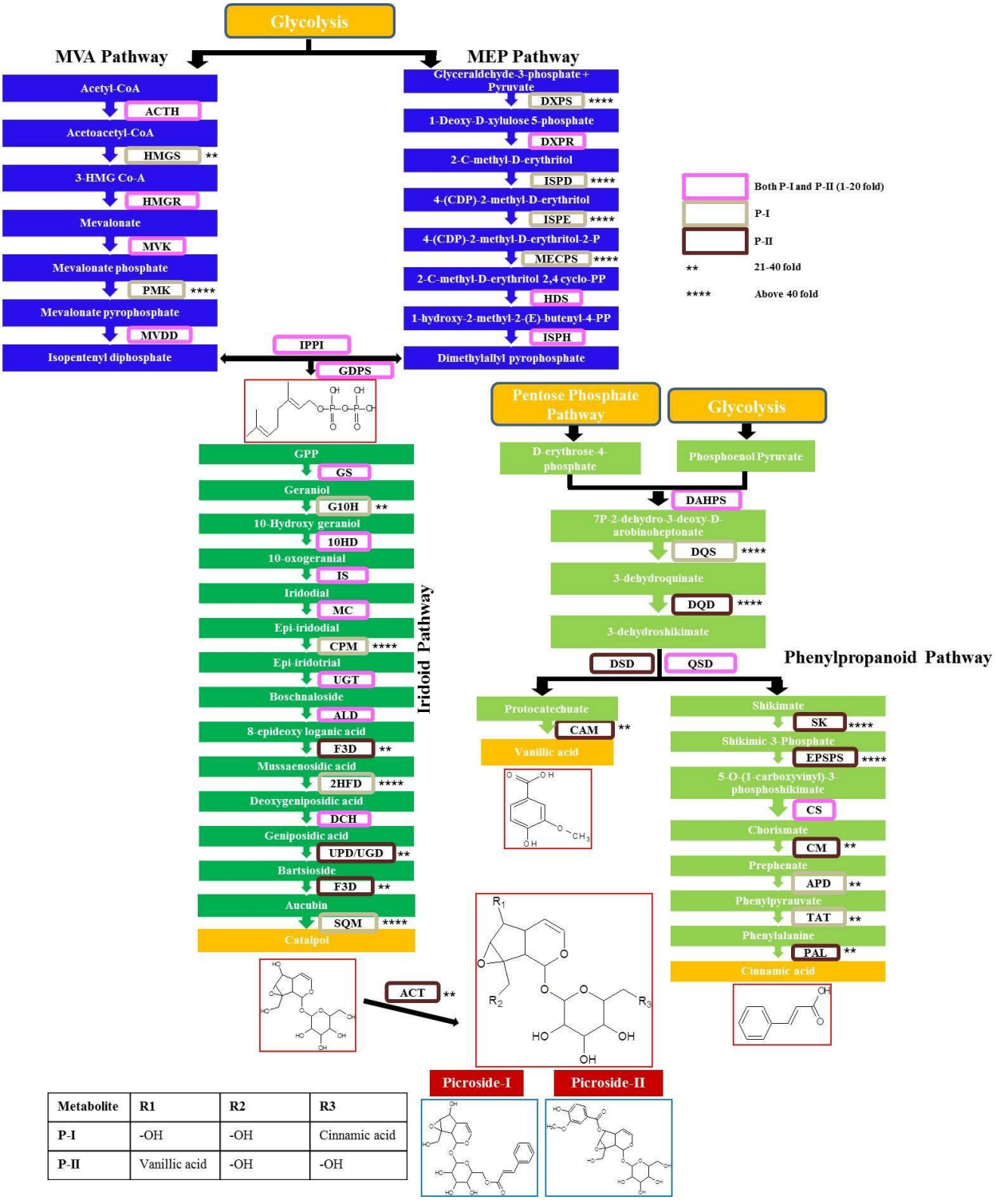


Figure 4.6 Modular architecture of Picroside-I and Picroside-II biosynthetic pathways and expression status of genes depicted as fold increase in *Picrorhiza kurroa*: MVA and MEP pathway genes data from Pandit et al. [33]

4.8 Expression status of genes in high versus low Picroside-I content genotypes of *P. kurroa*

The genes which showed above 10 folds transcript abundance in initial screening w.r.t. either Picroside-I or Picroside-II or both (including MEP/MVA pathway genes from our previous study) were checked for their expression status on shoot tissues of high versus low Picroside-I content strains of *P. kurroa*. A high Picroside-I content strain, PKS-1 (2.7%) and a low content strain, PKS-4 (0.38%) were used in comparative expression analysis. This resulted in the identification of seven genes, ISPD, DXPS, ISPE, PMK, 2HFD, EPSPS, SK from the complete biosynthetic pathway with ~5-57 folds higher transcript abundance in high content strain compared to low content strain of *P. kurroa* (Figure 4.7).

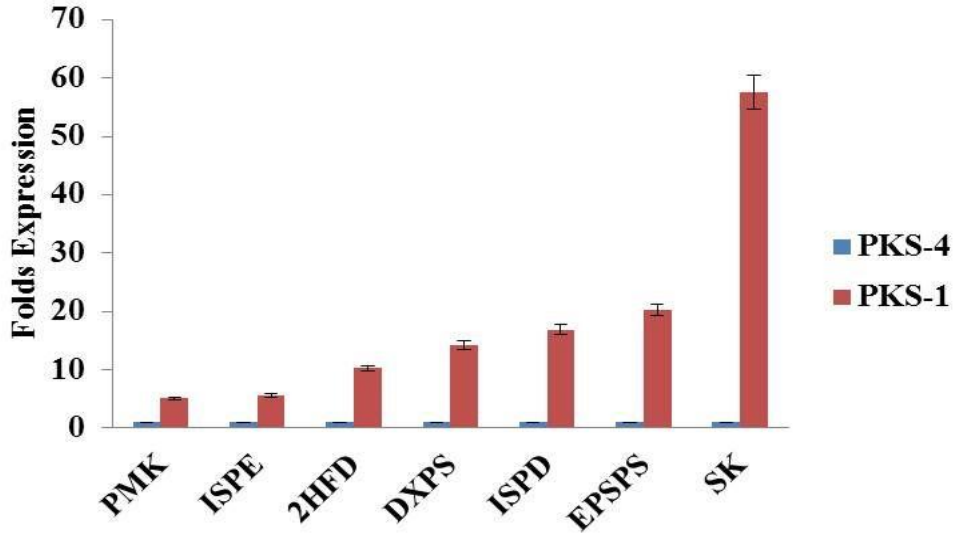


Figure 4.7 Expression pattern of key genes in shoot tissues of high (PKS-1, 2.7%) versus low (PKS-4, 0.38 %) Picroside-I content accessions of *Picrorhiza kurroa*

The expression of three genes of MEP pathway, DXPS, ISPE, ISPD, one gene of MVA pathway, PMK and one gene of iridoid pathway, 2HFD decreased from 57-160 folds to ~5-17 folds when compared for their relative transcript abundance between strains versus different tissue samples. The expression level of three genes of MEP pathway ISPD, DXPS and ISPE decreased from 160, 57 and 99 folds to 16.8, 14.2 and 5.6 folds, respectively in high content strain. The expression of PMK gene of MVA pathway decreased from 107.6 fold to 5.1 fold. 2HFD of iridoid pathway decreased from 130 to 10.2 fold (Table 4.6). The relative expression status of pathway genes between high versus low content strains thus provided a realistic association with the biosynthesis of picrosides rather other metabolites.

Table 4.6 Folds difference in expression status of key genes between A (FGS vs TCS) and B (PKS-1 vs PKS-4).

Gene	A	B	Fold Difference
PMK	107.66	5.10	-21.1
ISPE	99	5.63	-17.6
2HFD	130	10.25	-12.7
DXPS	57	14.25	-4.0
ISPD	160	16.86	-9.5
EPSPS	4.5	20.28	+4.5
SK	1.03	57.59	+55.9

FGS: Field grown shoots, **TCS**: Tissue cultured shoots grown at 25°C, **PKS-1**: High Picroside-I content strain, **PKS-4**: Low Picroside-I content strain

4.9 Effect of enzyme inhibitors on biosynthesis of Picroside-I

The inhibition profile of four enzyme inhibitors was assessed at 15th and 30th day of *in vitro* culturing of *P. kurroa* shoots by quantifying Picroside-I content (Figure 4.8) because cultured shoots are reported to accumulate only Picroside-I [22]. The best inhibitory concentrations were 5 µM for mevinolin, 150 µM for fosmidomycin, 4 mM for glyphosate and AOA. Mevinolin was found to be least effective in inhibiting Picroside-I biosynthesis as it showed maximum inhibition of only 17% and 11.6% at 15th and 30th day, respectively. Other inhibitors, fosmidomycin, glyphosate and AOA showed upto 64%-92% inhibition in Picroside-I content in comparison to control. All inhibitors showed higher inhibition at 30th day in comparison to 15th day, except mevinolin which unlike others showed higher inhibition at 15th day. At their best inhibitory concentrations, fosmidomycin, glyphosate and AOA showed a drastic reduction of 85.3%, 87.3% and 64.6% respectively in Picroside-I accumulation at 15th day and reduction of 90.6%, 92.9% and 63.4%, respectively at 30th day. Fosmidomycin, glyphosate and AOA were thus found to be potent inhibitors of Picroside-I biosynthesis.

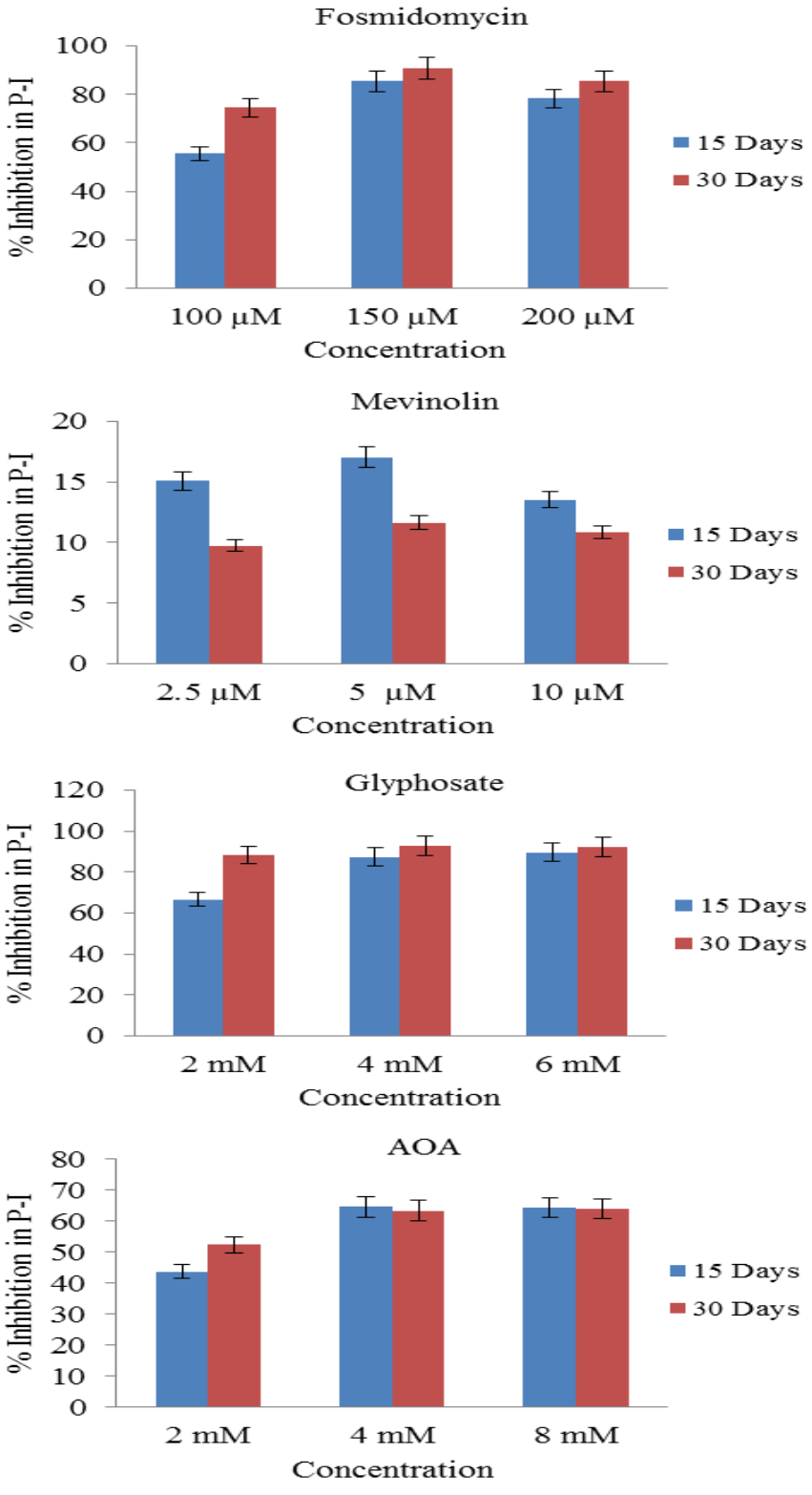


Figure 4.8 Effect of enzyme inhibitors on Picroside-I biosynthesis in *Picrorhiza kurroa*. The inhibitor profiles were analysed at 15th and 30th day of *in vitro* culturing at different inhibitor concentrations. The graphs show percentage inhibition in Picroside-I content w.r.t. control

4.10 Gene expression in response to inhibitors

To see the effect of inbitor treatments on target gene transcripts, expression analysis was carried out on inhibitor treated shoot tissues in comparison to untreated control for related genes (DXPR, HMGR, EPSPS and PAL) alongwith upstream and downstream genes (1 each). The expression analysis was carried out at the best inhibitory concentrations of 150 µM (fosmidomycin), 5 µM (mevinolin) and 4 mM (glyphosate and AOA) at 15^{th} and 30^{th} day of incubation. The inhibitors fosmidomycin, mevinolin, glyphosate and AOA did not show significant reduction in the transcript levels of their target steps (DXPR, HMGR, EPSPS and PAL, respectively) or upstream/downstream genes. Fosmidomycin showed 1.25-2.5 folds reduction in transcript levels for DXPS, DXPR and ISPD in comparison to control at both 15^{th} and 30^{th} day. Mevinolin, glyphosate and AOA also showed similar gene expression pattern with mevinolin 1-3.5 folds reduction in transcripts level for HMGS, HMGR and MVK, glyphosate 1-4.7 folds reduction for SK, EPSPS and CS and AOA 1-4 folds reduction for TAT, PAL and C4H in comparison to control (Figure 4.9).

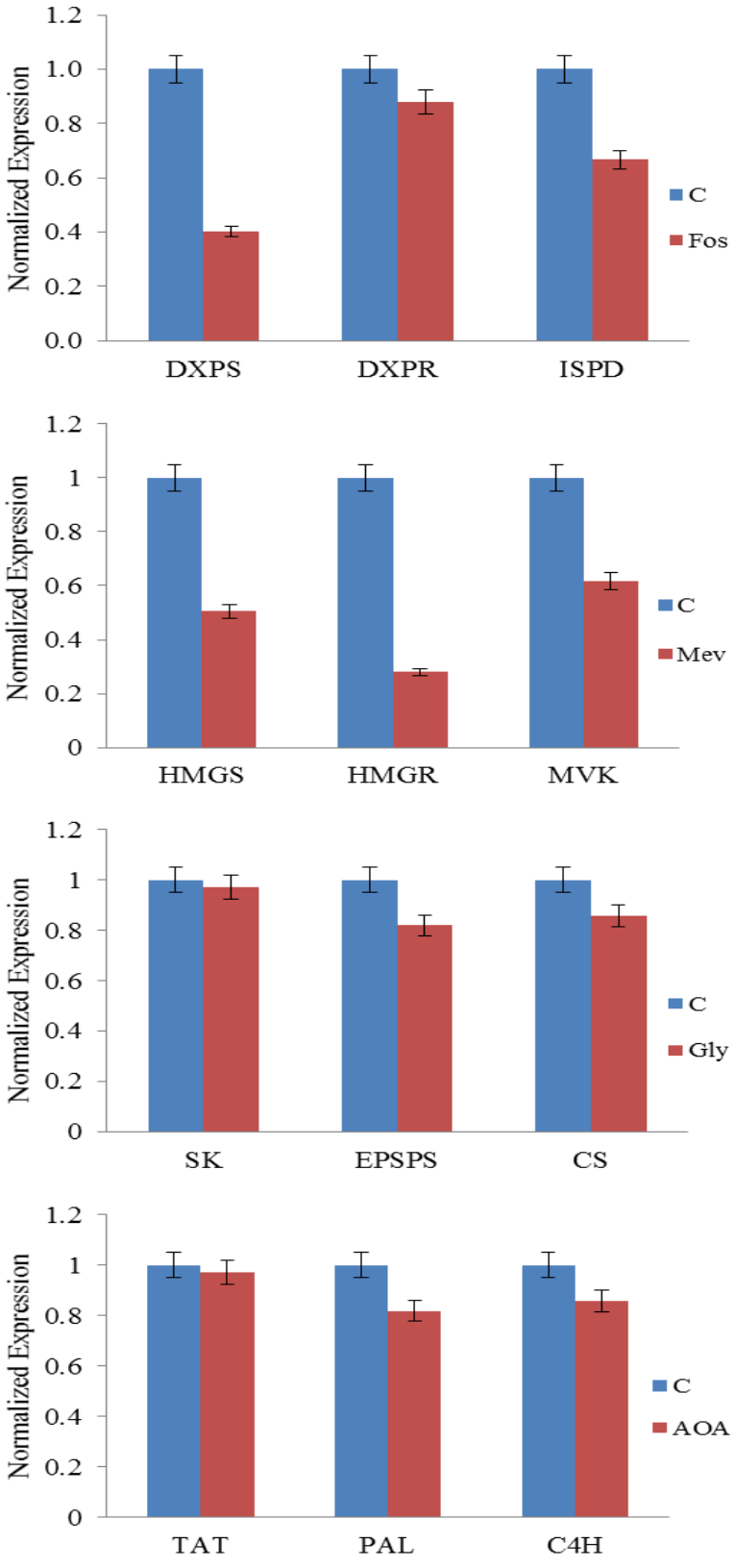


Figure 4.9 Effect of inhibitors, fosmidomycin (Fos), mevinolin (Mev), glyphosate (Gly) and AOA on transcript levels of target as well as upstream and downstream genes in Picroside-I biosynthesis in *Picrorhiza kurroa*

4.11 Occurrence and distribution of SSRs in *Picrorhiza kurroa*

Mining six *P. kurroa* transcriptomes (PKS-25, PKR-25, PKS-15, PKSS, PKSR, PKSTS) for SSRs revealed that the percentage of trinucleotide SSRs was highest (49.9%) followed by mono (24.3%), di (12.5%), tetra (5.8), hexa (4.7%) and penta (2.8%). A total of 21,748 SSRs were identified, 5136 in PKR-25, 4248 in PKS-15, 5754 in PKS-25, 1769 in PKSR, 2082 in PKSS and 2759 in PKSTS (Table 4.7). From the total SSRs identified in *P. kurroa* transcriptomes, 360 primer pairs were designed targeting different types of repeat motifs including mono, di, tri, tetra, penta and hexa nucleotide repeats for testing their amplification and polymorphisms on twenty six accessions of *P. kurroa*.

Table 4.7 Distribution of SSRs in six different transcriptomes of *P. kurroa*

Sample Name	Motif Length	Repeat Number									Total	%
		4	5	6	7	8	9	10	11	>11		
PKR-25	Mono	0	0	0	0	0	0	0	0	2000	2000	38.9
	Di	0	0	0	103	62	42	31	35	20	293	5.7
	Tri	0	774	296	207	124	65	61	200	290	2017	39.3
	Tetra	210	43	21	11	3	6	2	5	30	331	6.4
	Penta	153	37	14	5	6	3	0	1	0	219	4.3
	Hexa	172	65	22	7	1	7	1	1	0	276	5.4
	Total	535	919	353	333	196	123	95	242	2340	**5136**	
	%	10.4	17.9	6.9	6.5	3.8	2.4	1.8	4.7	45.6		
PKS-15	Mono	0	0	0	0	0	0	0	0	741	741	17.4
	Di	0	0	0	214	137	99	57	46	100	653	15.4
	Tri	0	1224	524	211	110	76	37	28	140	2350	55.3
	Tetra	194	29	7	8	0	0	0	5	7	250	5.9
	Penta	67	21	19	0	1	0	0	1	0	109	2.6
	Hexa	97	41	4	3	0	0	0	0	0	145	3.4
	Total	358	1315	554	436	248	175	94	80	988	**4248**	
	%	8.4	31.0	13.0	10.3	5.8	4.1	2.2	1.9	23.3		
PKS-25	Mono	0	0	0	0	0	0	0	0	1452	1452	25.2
	Di	0	0	0	270	200	129	66	72	101	838	14.6
	Tri	0	1476	630	281	107	49	51	90	103	2787	48.4
	Tetra	233	32	2	3	4	4	1	2	4	285	5.0
	Penta	68	27	20	1	0	0	1	0	0	117	2.0
	Hexa	193	63	9	9	1	0	0	0	0	275	4.8
	Total	494	1598	661	564	312	182	119	164	1660	**5754**	
	%	8.6	27.8	11.5	9.8	5.4	3.2	2.1	2.9	28.8		
PKSR	Mono	0	0	0	0	0	0	0	0	262	262	14.8
	Di	0	0	0	95	62	31	19	11	15	233	13.2
	Tri	0	554	246	116	32	25	9	20	24	1026	58.0
	Tetra	77	24	5	3	3	1	0	1	2	116	6.6
	Penta	33	3	3	0	0	0	0	0	0	39	2.2
	Hexa	62	24	5	1	1	0	0	0	0	93	5.3
	Total	172	605	259	215	98	57	28	32	303	**1769**	
	%	9.7	34.2	14.6	12.2	5.5	3.2	1.6	1.8	17.1		
PKSS	Mono	0	0	0	0	0	0	0	0	392	392	18.8

	Di	0	0	0	93	87	47	25	21	37	310	14.9
	Tri	0	559	225	118	52	52	17	18	33	1074	51.6
	Tetra	68	37	4	5	2	1	0	15	2	134	6.4
	Penta	36	9	7	0	1	0	0	0	0	53	2.5
	Hexa	82	20	3	7	7	0	0	0	0	119	5.7
	Total	186	625	239	223	149	100	42	54	464	**2082**	
	%	8.9	30.0	11.5	10.7	7.2	4.8	2.0	2.6	22.3		
PKSTS	Mono	0	0	0	0	0	0	0	0	441	441	16.0
	Di	0	0	0	136	90	48	35	40	34	383	13.9
	Tri	0	841	383	142	87	67	24	31	34	1609	58.3
	Tetra	109	20	5	1	3	3	0	3	1	145	5.3
	Penta	49	13	6	1	0	0	0	2	0	71	2.6
	Hexa	74	21	5	3	3	0	4	0	0	110	4.0
	Total	232	895	399	283	183	118	63	76	510	**2759**	
	%	8.4	32.4	14.5	10.3	6.6	4.3	2.3	2.8	18.5		

4.12 SSR amplification and polymorphism analysis

The 360 SSR primers designed from *P. kurroa* transcriptomes, were tested for amplification as well as extent of polymorphism on 26 accessions of *P. kurroa*. The list of 360 primers has been provided in Appendix-I (Table A1). Out of 361 SSRs, 35 (9.69%) primer pairs showed polymorphisms. Representative polymorphic gel pictures for SSR markers PKSS M15, PKSTS D1, PKSS D5, PKSTS M13 and PKSTS TT2 are shown in Figure 4.10. Out of 35 polymorphic SSRs (Table 4.8), the SSRs with mononucleotide repeat motifs showed highest (25.7%) level of polymorphisms followed by di (22.9%), hexa (22.9%), tetra (14.3%), penta (8.6%) and tri (5.7%). Di- and hexa-nucleotide SSRs showed same level of polymorphisms whereas trinucletide SSRs showed least polymorphism. By analysing the annotation of transcriptomic regions from which SSR primers were designed it was found that some of the polymorphic SSR markers were lying in the important genic regions such as LRR receptor like serine/threonine protein kinase, WRKY transcription factor, translation initiation factor eif4a and primary metabolism such as phosphofructokinase.

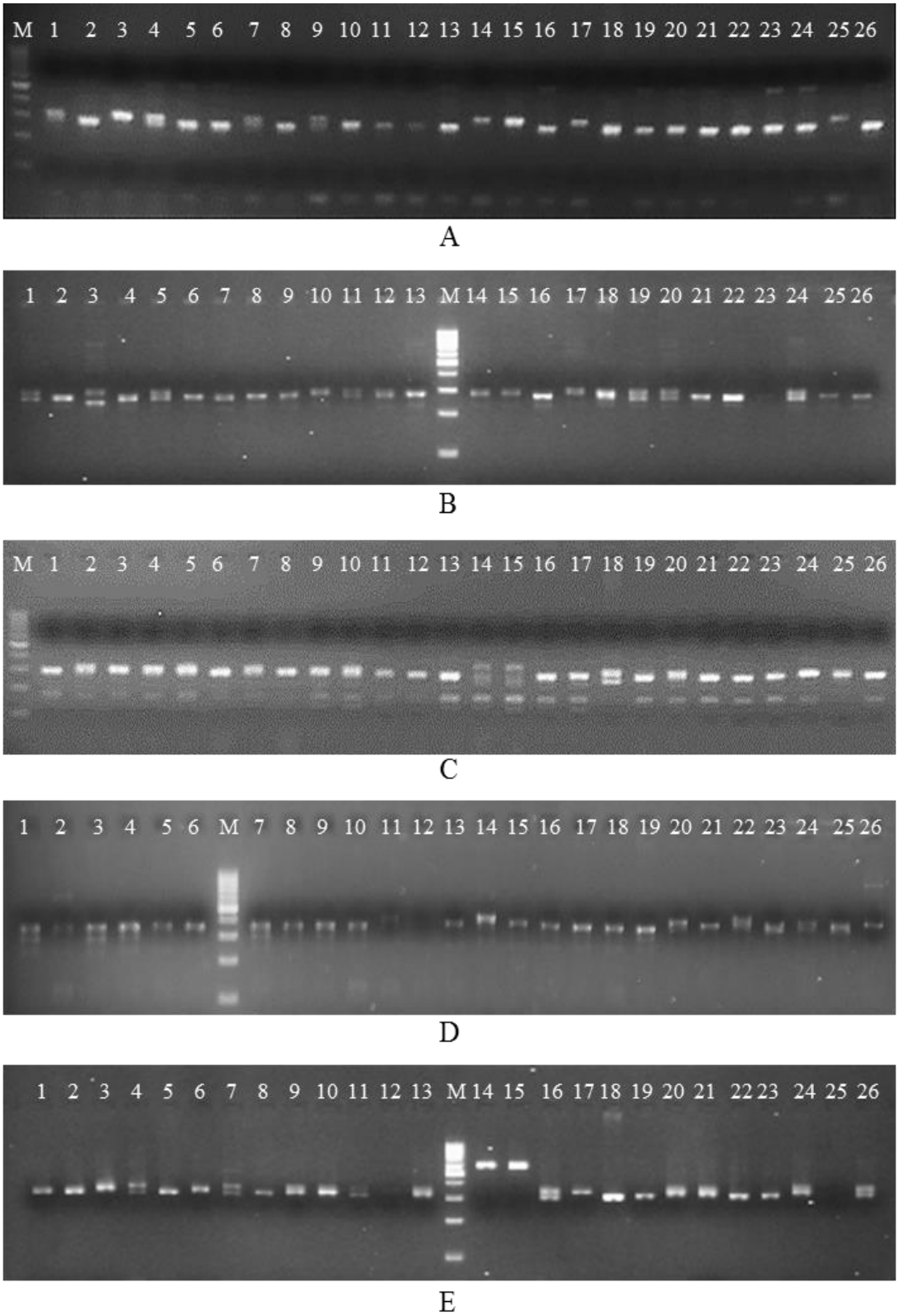


Figure 4.10 SSR markers showing polymorphism in 26 accessions (Lanes 1-26) of *P. kurroa*. M is 100bp DNA ladder. (A) PKSS M15 (B) PKSTS D1 (C) PKSS D5 (D) PKSTS M13 (E) PKSTS TT2

Table 4.8 Genic locations of SSRs which showed polymorphisms among accessions of *P. kurroa*

SSR locus	SSR Motif	Target locus annotation in transcriptome	Primer Sequence	Annealing Temp. (°C)
PKSTS-D1	$(CT)_{20}$	lrr receptor-like serine threonine-protein kinase fls2-like	5'-AATGTAGCTTGCTTTGGTGT-3' 5'-TTGAGTCATGAGTCATGGAA-3'	52
PKSTS-T2	$(TTC)_{11}$	lrr receptor-like serine threonine-protein kinase fls2-like	5'-GGTAATTTCCCCTTCTGTTT-3' 5'-TATCCAAATCGAAAGGAAGA-3'	49
PKSTS-TT2	$(ACTC)_3$	Hypothetical chloroplast rf68	5'-TACGCTAATGCAGTTCTTCA-3' 5'-CGTATTGGTACAACTACGCA-3'	53
PKSTS-TT13	$(CCAA)_3$	Bel1-like homeodomain protein 1-like	5'CCTAAGAGAAGAACGAGCAA3' 3'TTTTTCCTAGTTTTCCTGCA5'	51
PKSTS-P1	$(TTATT)_4$	lrr receptor-like serine threonine-protein kinase fls2-like	5'-ACGAGTTTTCTGCAGATGAT-3' 5'-AACCAAATTGAACACACACA-3'	51
PKSTS-H8	$(CTCCAG)_4$	e3 ubiquitin-protein ligase upl1-like	5'-GTTTTATTCGTGTTTACCGG-3' 5'-TAACATACCGACCAGGATTC-3'	50
PKSTS-H11	$(TCGAGC)_3$	PREDICTED: uncharacterized protein LOC100267936 [Vitis vinifera]	5'-TCGTCATACCTAGAGCCTTC-3' 5'-TCTCACACAACACACACACA-3'	54
PKR25-H7	$(AAAAAT)_3$	Retroelement pol poly	5'-GTGTCGACGTCTCCTACAAT-3' 5'-TTACTTGTCGACCGTGTACA-3'	54
PKR25-H11	$(TAAAAA)_3$	fin and gill-specific type-1 keratin	5'-TCAGCAAACGGATTTTAGAT-3' 5'-CCCAAAAAGTCTGAAACAAC-3'	50
PKSS-M15	$(A)_{14}$	s-adenosylmethionine synthase-like isoform 1	5'-TCAACTCCTGAAGATCAGCT-3' 5'-GGTAGACTCGTGCACCATAT-3'	52
PKSS-D5	$(AG)_{10}$	wrky transcription factor	5'-AAAGGAGTTTGGCTCTAGCT-3' 5'-TACCCAGTACTACGGAAAGG-3'	51
PKS15-D14	$(CT)_{14}$	No blast hit	5'-ACACAAGGTATGCAAGCTTT-3' 5'-GTTCATTGATCCATGACATG-3'	51
PKSTS-M1	$(A)_{14}$	Glutamine synthetase	5'-CTTTCAAGATTTATCGACCG-3' 5'-ACGACAAAAAGAACTCCTGA-3'	50
PKSTS-M4	$(A)_{12}$	Cytochrome c oxidase subunit 2	5'-AGAGAATAGGCCTGTCACCT-3' 5'-TAGAACCACGATGATTCACA-3'	53
PKSTS-M7	$(A)_{12}$	Cation-transporting atpase protein binding	5'-TTAACTTTCAATACCCCCAA-3' 5'-AGAAGCTGATACAGCGAAAG-3'	51
PKSTS-M8	$(T)_{12}$	Translational initiation factor eif-4a	5'-TTGATGCACACAAGAGTGAT-3' 5'-CTATTCGGTTGAGCATAAGC-3'	52
PKSTS-M10	$(T)_{12}$	Calcium-transporting atpase plasma membrane-type-like	5'-TTTAGACTTGATAGCTGCCC-3' 5'-ACTGATAGAGCGTGAGAGGA-3'	53

PKSTS-M13	$(T)_{12}$	f-box protein skip14-like	5’-TTTTCTTGGGTTTAGTTGGA-3’ 5’-AAGATGTGAGGTAGGTGGTG-3’	51
PKSTS-D3	$(AC)_9$	rna-dependent rna polymerase	5’-GGACCTTAGCTCCTCAAACT-3’ 5’-GTTTTCCGTGGTGTGTTTAT-3’	52
PKSTS-TT15	$(TGGT)_3$	Nucleolar protein 14-like	5’CTCATTATGCAGTTGGTGTG3’ 3’CAGCTAAGCATATGGAATCC5’	51
PKSTS-P9	$(TGGTG)_4$	Probable histone-lysine n-methyltransferase atxr3-like	5’-TGCTTTCGACTAGCTTCTTC-3’ 5’-CGTCTATCAGAATAATCGGC-3’	51
PKSTS-H5	$(CGGGAA)_8$	p30 dbc	5’-AAGGTCTTCTTTACGCTCCT-3’ 5’-CTATCTCTTTCCCGTACCCT-3’	53
PKSTS-H12	$(AAATCC)_4$	Methyltransferase pmt3	5’-TTCATTTTGGCACATGAGTA-3’ 5’-TGCAAACACTCAAACTTTAACT-3’	49
PKSTS-H15	$(GGTGGA)_3$	Zinc finger ccch domain-containing protein	5’-GAATTCGCTTGACGAATTAC-3’ 5’-TTTGATTCCTTAAACAGGGA-3’	49
PKR25-D4	$(AT)_7$	Photosystem ii reaction center protein m	5’-TTTTTCAATGAAATATCGTGAA-3’ 5’-GGATTTGAACCGATGACTTA-3’	48
PKR25-D13	$(AT)_8$	Reverse transcriptase	5’-TTTAGAGGATTTGGTGCAAT-3’ 5’-TTGGGTAAGAGTTGCAAGTT-3’	51
PKR25-T1	$(AAT)_5$	Tetracycline resistance protein	5’-CAAATATTGGGACGATATGC-3’ 5’-CCCGTCAAGTCTCTCTACAT-3’	51
PKR25-TT2	$(GTTT)_3$	Ribosomal protein l20	5’-ATCGTTGTTGTTTCAAGGTC-3’ 5’-TCCTTTCCTTGAACTTGAAA-3’	50
PKR25-P12	$(AGAAA)_3$	Copia protein	5’-TGGGAATGATCGAAATAAAC-3’ 5’-TCCTGATATCCACTTCTGCT-3’	50
PKR25-H8	$(TTTCTT)_3$	Response regulator receiver protein	5’-AGAGGCCGTAAGAGAGTTCT-3’ 5’-CTCCAATTCAACCATTGATT-3’	52
PKS15-M1	$(T)_{48}$	Photosystem ii 47 kda protein	5’-TCAATAGACTGCTAAAGCCC-3’ 5’-TCGTCTTTTTGGTCTCATTT-3’	53
PKS15-M15	$(T)_{12}$	No blast hit	5’-ATCACTTATTTTGTCGCGTT-3’ 5’-CGCCCAAAAAGAAAATTATA-3’	50
PKS15-TT1	$(CAAT)_3$	ycf1 protein	5’-GTGGATTGATGACGAGAGAT-3’ 5’-AATTCTTCTCTTTACCTTACCAAA-3’	51
PKSR-D5	$(AT)_8$	6-phosphofructokinase 3 like	5’-CAAATTTCGAAGTACTTGGG-3’ 5’-CAAAAGCTCCCATCTAAATG-3’	52
PKSTS-D2	$(TG)_8$	lrr receptor-like serine threonine-protein kinase fls2-like	5’-AATGTAGCTTGCTTTGGTGT-3’ 5’-AAGAGAAGTTTTGCGTCAAC-3’	52

4.13 Genetic diversity and cluster analysis of *P. kurroa* accessions

Genetic diversity analysis was done using Popgene. Among all accessions Nei's genetic diversity (h) ranged from 0.26 to 0.5 with a mean value of 0.39 (St. Dev. 0.226). Shannon's information index (I) ranged from 0.16 to 0.69 with mean value of 0.58 (St. Dev. 0.109). Summary of genic variation statistics for all loci is given in Table 4.9.

Table 4.9 Summary of genetic variation statistics for all polymorphic SSR markers

Locus	Sample size	ne	h	I
PKSTS-D1	26	1.5505	0.355	0.5402
PKSTS-T2	26	1.6488	0.3935	0.5825
PKSTS-TT2	26	1.352	0.2604	0.4293
PKSTS-TT13	26	1.8989	0.4734	0.6663
PKSTS-P1	26	1.827	0.4527	0.645
PKSTS-H8	26	1.9882	0.497	0.6902
PKSTS-H11	26	1.4506	0.3107	0.4896
PKR25-H7	26	1.827	0.4527	0.645
PKR25-H11	26	1.4506	0.3107	0.4896
PKSS-M15	26	2.000	0.500	0.6931
PKSS-D5	26	1.6488	0.3935	0.5825
PKS15-D14	26	1.827	0.4527	0.645
PKSTS-M1	26	1.8989	0.4734	0.6663
PKSTS-M4	26	1.352	0.2604	0.4293
PKSTS-M7	26	1.9538	0.4882	0.6813
PKSTS-M8	26	1.5505	0.355	0.5402
PKSTS-M10	26	2.000	0.500	0.6931
PKSTS-M13	26	1.0799	0.074	0.163
PKSTS-D3	26	1.6488	0.3935	0.5825
PKSTS-TT15	26	1.6488	0.3935	0.5825
PKSTS-P9	26	1.827	0.4527	0.645
PKSTS-H5	26	1.7423	0.426	0.6172
PKSTS-H12	26	1.9538	0.4882	0.6813
PKSTS-H15	26	1.352	0.2604	0.4293
PKR25-D4	26	1.8989	0.4734	0.6663
PKR25-D13	26	1.7423	0.426	0.6172
PKR25-T1	26	1.6488	0.3935	0.5825
PKR25-TT2	26	1.9538	0.4882	0.6813
PKR25-P12	26	1.352	0.2604	0.4293
PKR25-H8	26	1.6488	0.3935	0.5825

PKS15-M1	26	1.5505	0.355	0.5402
PKS15-M15	26	1.7423	0.426	0.6172
PKS15-TT1	26	1.7423	0.426	0.6172
PKSR-D5	26	1.6488	0.3935	0.5825
PKSTS-D2	26	1.827	0.4527	0.645
Mean		1.6924	0.3973	0.582
St. Dev.		0.2262	0.0926	0.1089

POPGENE was used to calculate effective number of alleles (ne), Nei's genetic diversity (h) and Shannon's information index (I)

Observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}) and polymorphism information content (PIC) were calculated using Cervus software. H_{obs} ranged from 0.115 to 1.000 with an average of 0.597 and H_{exp} ranged from 0.113 to 0.634 with an average of 0.452. The PIC values for polymorphic SSRs varied from 0.11 to 0.55 with an average of 0.39. The highest PIC value was for SSR marker PKSTS-P9 whereas PKSTS-P1 showed the least PIC value. The PIC values for polymorphic markers alongwith number of alleles are given in Table 4.10.

Table 4.10 PIC values, H_{obs} and H_{exp} at each SSR locus

S. No.	**SSR locus**	**Sample size**	**No. of alleles**	**H_{obs}**	**H_{exp}**	**PIC**
1	PKSTS-D1	26	3	0.538	0.411	0.336
2	PKSTS-T2	26	3	0.654	0.480	0.402
3	PKSTS-TT2	26	2	0.154	0.145	0.132
4	PKSTS-TT13	26	3	0.885	0.603	0.525
5	PKSTS-P1	26	3	0.115	0.112	0.107
6	PKSTS-H8	26	3	0.769	0.562	0.472
7	PKSTS-H11	26	3	0.269	0.245	0.224
8	PKR25-H7	26	3	0.346	0.302	0.270
9	PKR25-H11	26	3	0.462	0.388	0.348
10	PKSS-M15	26	3	0.385	0.335	0.303
11	PKSS-D5	26	3	0.923	0.540	0.424
12	PKS15-D14	26	3	0.692	0.509	0.420
13	PKSTS-M1	26	3	0.500	0.539	0.472
14	PKSTS-M4	26	3	0.231	0.212	0.193
15	PKSTS-M7	26	3	0.72	0.571	0.498
16	PKSTS-M8	26	3	0.654	0.504	0.426
17	PKSTS-M10	26	3	0.731	0.582	0.504
18	PKSTS-M13	26	3	0.615	0.482	0.425
19	PKSTS-D3	26	3	0.269	0.242	0.217
20	PKSTS-TT15	26	3	0.962	0.627	0.546

21	PKSTS-P9	26	3	1.000	0.634	0.551
22	PKSTS-H5	26	3	0.577	0.461	0.409
23	PKSTS-H12	26	3	0.423	0.363	0.327
24	PKSTS-H15	26	3	0.923	0.578	0.482
25	PKR25-D4	26	3	0.962	0.612	0.524
26	PKR25-D13	26	3	0.808	0.542	0.450
27	PKR25-T1	26	3	0.385	0.435	0.384
28	PKR25-TT2	26	3	0.885	0.603	0.525
29	PKR25-P12	26	3	0.538	0.426	0.366
30	PKR25-H8	26	3	0.615	0.456	0.376
31	PKS15-M1	26	3	0.500	0.405	0.352
32	PKS15-M15	26	3	0.654	0.555	0.484
33	PKS15-TT1	26	3	0.846	0.586	0.509
34	PKSR-D5	26	3	0.462	0.386	0.343
35	PKSTS-D2	26	3	0.462	0.386	0.343
Mean			2.97	0.597	0.452	0.391

Unbiased clustering of accessions was done by using STRUCTURE. This model is based on MCMC (Monte Carlo Markov Chain). STRUCTURE provides a model-based approach to infer population structure by using SSR marker dataset to identify k clusters to which it then assigns each individual. The results obtained from structure were analysed using an online tool Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/). The optimum k value was found to be 2. This value signifies the number of clusters in which the individuals can be dived in. The accessions were grouped into two clusters (Figure 4.11).

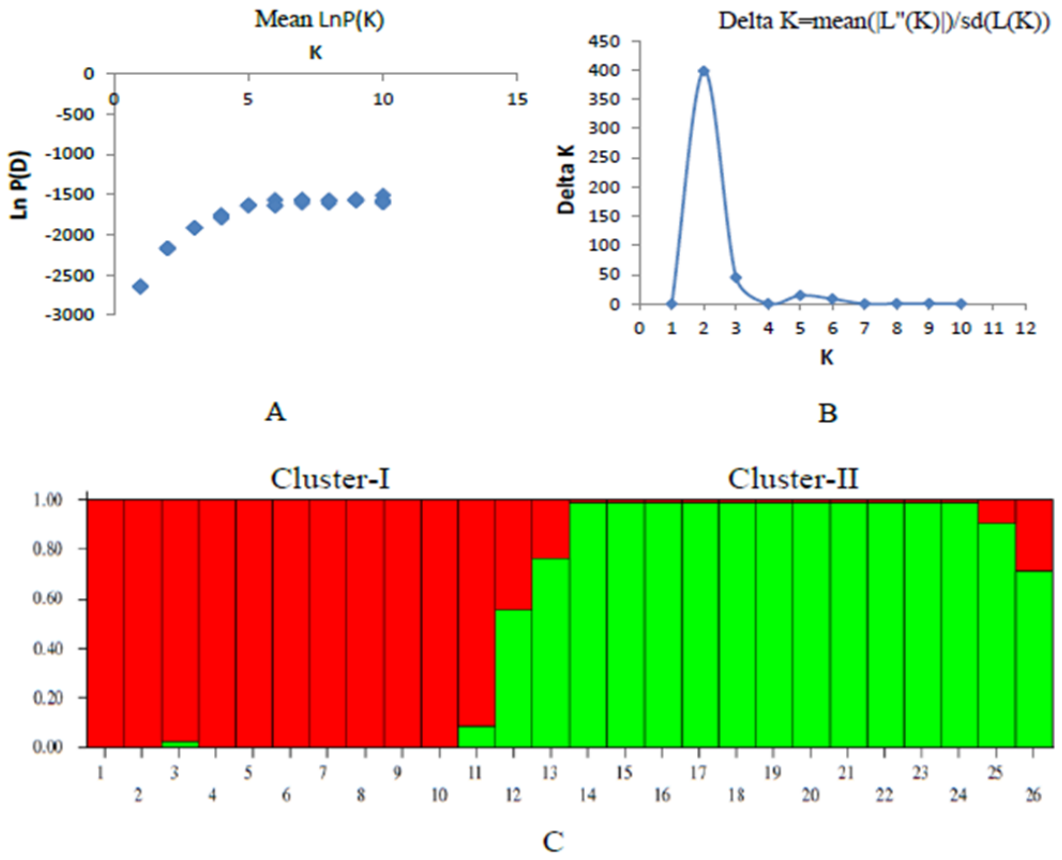


Figure 4.11 (A) The relationship between number of clusters (K) and the estimated likelihood of data (LnP(D)). A model based clustering of 26 accessions using STRUCTURE without prior knowledge about the populations and under an admixed model calculated that LnP(D) was greatest when K = 2. **(B)** The relationship between K and ΔK., i.e. ΔK is reaches its maximum when K =2, suggesting that all accessions fall into one of the 2 clusters **(C)** Population structure analysis of 26 accessions using STRUCTURE.

Phylogenetic relationships among different accessions of *P. kurroa* were inferred by Darwin using DICE similarity coefficient to verify the clustering pattern obtained from structure. This is a biased clustering method which is based on genetic distance similarity. Cluster analysis generated a dendrogram for *P. kurroa* accessions which represented the overall genetic relationship among accessions (Figure 4.12). The clustering pattern obtained in Darwin was similar to unbiased clustering through structure. The accessions were divided into two major clusters. Accessions having Picroside-I content ranging from 0.9-2.7% in fresh shoots were grouped in cluster I and accessions having Picroside-I content from 0-0.9% were grouped in cluster II. Majority of the high content accessions were lying in cluster I and low content accessions in cluster II, intermediate content accessions were

distributed in both the clusters. PKS-1 and PKS-2 having high Picroside-I contents of 2.7% and 1.8% respectively, were placed together in cluster I in the dendrogram which shows that these two high content accessions are similar at the genetic level. Similarly, in cluster II, PKS-19 and PKS-20 having Picroside-I contents of 0.92% and 0.86% respectively, were placed together in the dendrogram. The twenty six accessions were grouped according to their respective Picroside-I contents in the dendrogram, however, there were few exceptions in both the clusters, for example, PKS-4 having a very low Picroside-I content of 0.38% was placed and cluster I and PKS-26 with high Picroside-I content of 1.6% was grouped in cluster II with low content accessions.

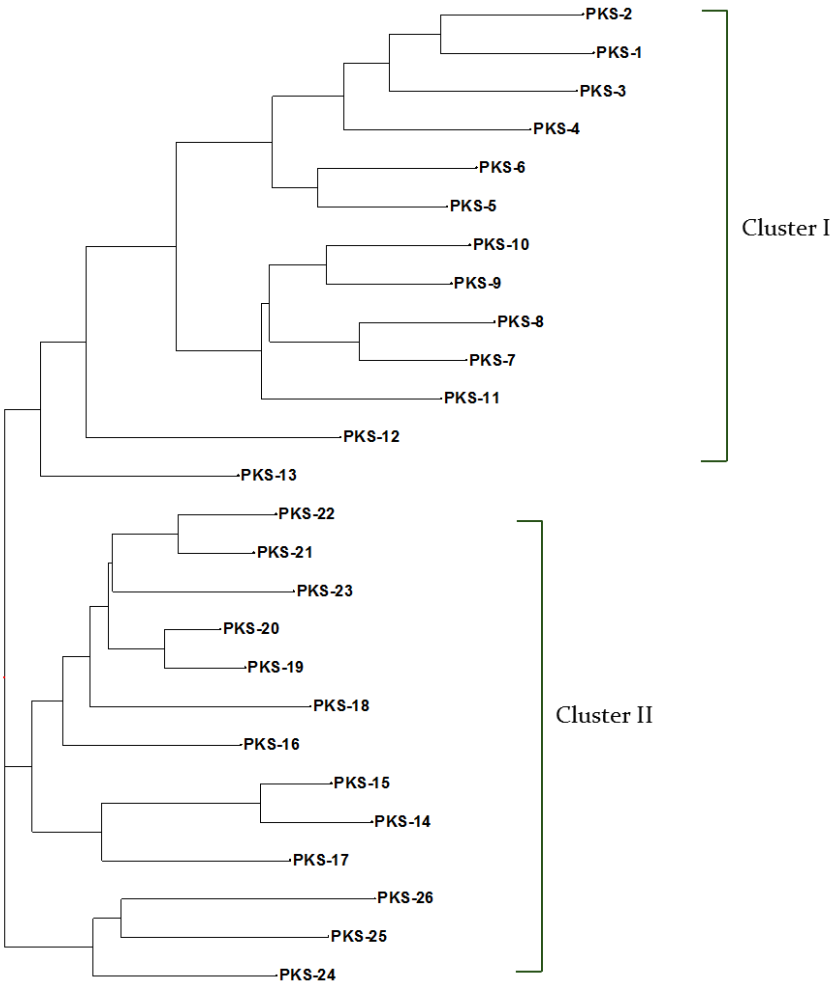


Figure 4.12 Cluster analysis (Neighbour joining) dendrogram generated through DARwin for 26 accessions of *P. kurroa*, based on the Dice similarity coefficient matrix. The accessions were grouped into two clusters, Cluster I with accessions having Picroside-I (0.9-2.7%) in fresh shoots and Cluster II with accessions having Picroside-I (0-0.9%)

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 infloroscence 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 isomer 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.

[33] 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, ISPH, 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 rosues* and its role in iridoid biosynthesis was supported by biochemical assays, gene silencing, co-expression analysis and localization studies [154]. Monoterpene cylase (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 dehydrogenae 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/hydoxylase (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 H_2O 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 bartsioside which involves removal of CO_2 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-xylose 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 palce through acyl group transfer. This acylation reaction can be catalyzed by acyltranferase (ACT) enzyme. An acyltransferase known as anthocyanin acyltransferases 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 preferably 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 roseues* [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 monoterpene 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 upto 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 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].

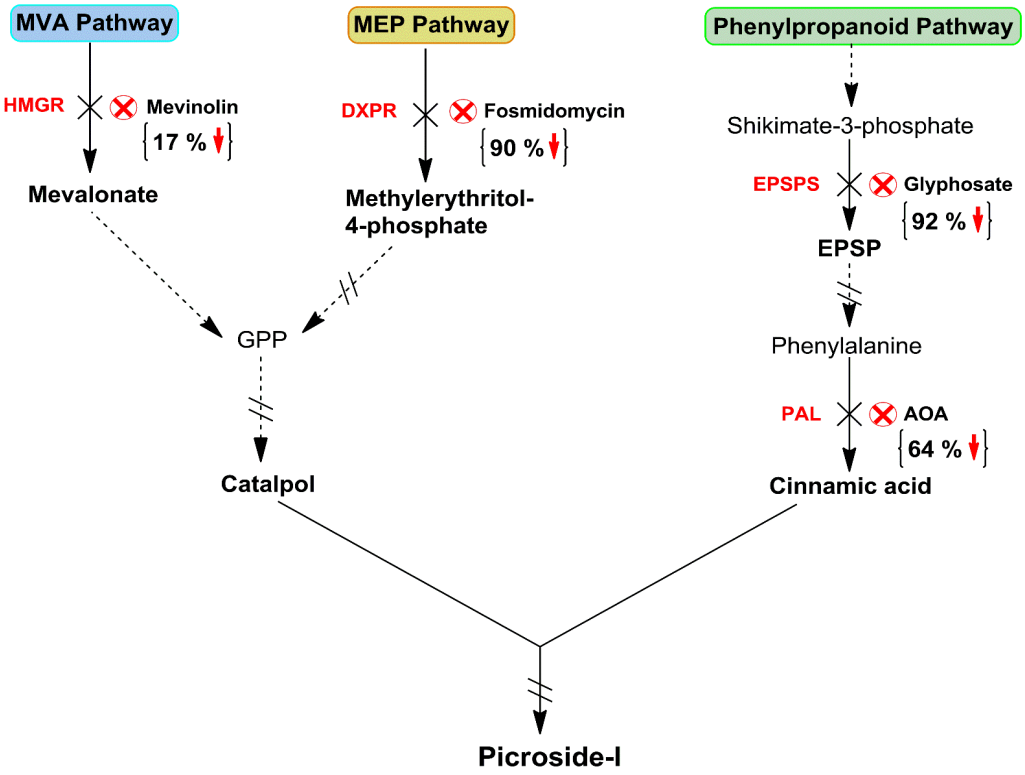


Figure 5.1 Representation of effect of different enzyme inhibitors on Picroside-I accumulation in *Picrorhiza kurroa*. Enzyme inhibitors point towards MEP route as a major contributor for picrosides biosynthesis

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 pathays 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 alongwith 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* trasncriptome. 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 trasncriptomic 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.

CONCLUSION AND FUTURE PROSPECTS

The outcome of this study necessitates the planning of conservation strategies for this important medicinal herb. The genetically stable superior accessions having high picrosides content can be used for mass cultivation at lower altitudes to provide high quality raw material. Further, the knowledge of complete pathway and corresponding genes would be helpful in understanding molecular basis of picrosides biosynthesis as well as planning genetic improvement strategies for enhancing picrosides content in *P. kurroa*. Inhibitor experiments revealed that MEP pathway is a major contributor of GPP for picrosides in *P. kurroa* and picrosides biosynthesis is being regulated at various control points in different modules of the biosynthetic route. Therefore, multiple genes need to be targeted for enhancing picrosides content. This is the first report wherein key genes have been identified in picrosides biosynthetic pathway which can have potential implications in molecular breeding and metabolic engineering after further functional validation using approaches like enzyme assays and gene silencing. Identification and analysis of transcription factors, transporters and other regulatory elements associated with key genes would also be useful in designing a suitable genetic intervention strategy for the enhanced production of picrosides in *P. kurroa*. The information on SSR markers would be helpful in the development of DNA diagnostics for the authentication of quality plant material.

SUMMARY

Quality assessment of *P. kurroa* raw material from herbal drug markets for major chemical constituents, Picroside-I and Picroside-II has highlighted variation in quality of rhizome samples being used by herbal drug industries. Therefore, analysis of quality of plant material is necessary so that appropriate plant material is selected for preparation of herbal drug formulations with desired pharmacological efficacy. Further, 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. In our previous studies, 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]. In present study, estimation of picrosides contents in twenty six accessions of *P. kurroa* from different geographical locations in Himachal Pradesh revealed that a wide range of natural variation exists for major compounds in *P. kurroa*. It also highlighted the importance of selection of appropriate tissue for assessing extent of genetic variation contributing to variation in chemotypes. High picrosides content accessions have been identified which can be recommended for mass cultivation for the production of quality raw material. Next, the elucidation of complete biosynthetic pathways of Picroside-I and Picroside-II alongwith corresponding genes at each enzymatic step was done because any genetic intervention to enhance picrosides content would require in depth knowledge on complete biosynthetic machinery and the genes involved in it. The NGS transcriptomes generated from different *P. kurroa* tissues and information from KEGG database were helpful in the elucidation of biosynthetic pathways. The correct functional paralog for each biosynthetic pathway gene was identified as multiple paralogs existed for pathway genes in the transcriptomes. Expression analysis of all genes of the biosynthetic pathway was done vis-à-vis Picroside-I and Picroside-II contents in different tissues of *P. kurroa* to identify important genes involved in their biosynthesis. The expression analysis in different tissues showed increased transcripts for twenty genes, which were further shortlisted to seven genes, ISPD, DXPS, ISPE, PMK, 2HFD, EPSPS and SK through expression analysis in shoot tissues of high versus low Picroside-I content accessions of *P. kurroa*. Further, the major contribution of MEP pathway in picrosides biosynthesis was ascertained through inhibitor studies using enzyme inhibitors fosmidomycin and mevinolin inhibiting the regulatory steps of MEP and MVA pathways, respectively. The importance of other module

i.e. phenylpropanoid pathway in picrosides biosynthesis was also inferred using enzyme inhibitors glyphosate and aminooxyacetic acid. Inhibitor treatments resulted in an inhibition of 17%-92% in Picroside-I accumulation. 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. Cluster analysis of *P. kurroa* accessions was also done using a software Darwin to infer genetic relationships of twenty six accessions. It was revealed that *P. kurroa* accessions from different geographical locations are not genetically highly diverse as indicated by low levels of polymorphisms.

This study is an initial endeavour towards elucidation of complete biosynthetic pathway of picrosides in *P. kurroa*. The biosynthetic pathway of Picroside-I and Picroside-II alongwith enzymatic steps is being reported for the first time in *P. kurroa*. The knowledge of complete pathway and corresponding genes would be helpful in understanding molecular basis of picrosides biosynthesis as well as planning genetic improvement strategies for enhancing picrosides content in *P. kurroa*. Expression analysis of the biosynthetic pathway genes in relation to picrosides content and identification of key genes revealed that picrosides biosynthesis is being regulated at various control points in different modules of the biosynthetic route. The major contribution of plastidial MEP pathway for picrosides biosynthesis was inferred through inhibitor experiments. This is the first report wherein key genes have been identified in picrosides biosynthetic pathway which can have potential implications in molecular breeding and metabolic engineering after further functional validation using approaches like enzyme assays and gene silencing. It is also concluded that multiple genes of the biosynthetic pathway need to be targeted for enhanced production of picrosides. Moreover, the information on SSR markers would be further helpful in the development of DNA diagnostics for the authentication of quality plant material. This research work has provided initial leads that can be taken forward to carry out any genetic improvement strategies for enhancing picrosides content in *P. kurroa*.

APPENDIX

Table A1 List of SSR primers designed from six transcriptomes of *P. kurroa* for testing amplification and polymorphism on twenty six accessions

S. No.	Primer	Repeat motif with number	Nucleotide Sequence	Annealing Temperature (°C)
1	PKSTS M1	$(A)_{14}$	5'-CTTTCAAGATTTATCGACCG-3' 5'-ACGACAAAAAGAACTCCTGA-3'	50
2	PKSTS M2	$(A)_{12}$	5'-CTACCTGCTTCTACGGAAGA-3' 5'-GCTATGCTATTGCCCTACTG-3'	53
3	PKSTS M3	$(A)_{14}$	5'-CTTGGAGATCCGACTACAAG-3' 5'-TTTAGGTGGTTCTCGAAAAA-3'	51
4	PKSTS M4	$(A)_{12}$	5'-AGAGAATAGGCCTGTCACCT-3' 5'-TAGAACCACGATGATTCACA-3'	53
5	PKSTS M5	$(A)_{14}$	5'-ACAAAGACAACAGACGCTCT-3' 5'-GATTCGCAAGAATTGAGAAG-3'	52
6	PKSTS M6	$(T)_{12}$	5'-CCGGAAATACTAGGCCTACT-3' 5'-CGATTGTCATAGGTATCTTTCA-3'	51
7	PKSTS M7	$(A)_{12}$	5'-TTAACTTTCAATACCCCCAA-3' 5'-AGAAGCTGATACAGCGAAAG-3'	51
8	PKSTS M8	$(T)_{12}$	5'-TTGATGCACACAAGAGTGAT-3' 5'-CTATTCGGTTGAGCATAAGC-3'	52
9	PKSTS M9	$(T)_{12}$	5'-CTATGTCACCATGCATGAAC-3' 5'-CTTTGTAGTCCATTCTTGGG-3'	51
10	PKSTS M10	$(T)_{12}$	5'-TTTAGACTTGATAGCTGCCC-3' 5'-ACTGATAGAGCGTGAGAGGA-3'	53
11	PKSTS M11	$(T)_{14}$	5'-GGGACTCTCTTCCTGTTTCT-3' 5'-AAGCTTTCAACCACAAGAAA-3'	52
12	PKSTS M12	$(T)_{16}$	5'-TCTAATATTCCTGCTGGGAA-3' 5'-AGTCACTCCTAGCTGCTTTG-3'	52
13	PKSTS M13	$(T)_{12}$	5'-TTTTCTTGGGTTTAGTTGGA-3' 5'-AAGATGTGAGGTAGGTGGTG-3'	51
14	PKSTS M14	$(T)_{12}$	5'-TAGGTGACACCATGTTCTCA-3' 5'-AGGTTGGTTAGTCACGAGAA-3'	53
15	PKSTS M15	$(A)_{14}$	5'-TTTCCACTTGGAAGACATTC-3' 5'-AGGAAGTGGACACTTGACAC-3'	52
16	PKSTS D1	$(CT)_{20}$	5'-AATGTAGCTTGCTTTGGTGT 5'-TTGAGTCATGAGTCATGGAA	52
17	PKSTS D2	$(TG)_{8}$	5'-AATGTAGCTTGCTTTGGTGT-3' 5'-AAGAGAAGTTTTGCGTCAAC-3'	52
18	PKSTS D3	$(AC)_{9}$	5'-GGACCTTAGCTCCTCAAACT-3' 5'-GTTTTCCGTGGTGTGTTTAT-3'	52
19	PKSTS D4	$(TC)_{8}$	5'-GAACTTAAGTGAGCGGAAAA-3' 5'-AAATTGAGAAATATGGCTGC-3'	50
20	PKSTS D5	$(CT)_{12}$	5'-ACAGGTTCTCTCACGAACAC-3' 5'-CAGCAATGAATCTCAAATGA-3'	51
21	PKSTS D6	$(AC)_{10}$	5'-GAAACAATTTGGATTTAGCG-3' 5'-CAACATCACCACATTCAAAC-3'	49
22	PKSTS D7	$(AG)_{7}$	5'-TTTTGACGTGTGTCAAGTGT-3' 5'-AGAGAAGCCTATCACGTGAA-3'	53
23	PKSTS D8	$(GA)_{14}$	5'-TTCTCGTGGTAAGCTCTGAT-3' 5'-GCCCTCTCTCTCTCTCTCTC-3'	54
24	PKSTS D9	$(TC)_{12}$	5'-TCTGCGGTTGAAATTCTACT-3' 5'-ACTAAAATTTGTGCCTTGGA-3'	51

25	PKSTS D10	$(TG)_{11}$	5’-GACTACGAACTTCTTGTGGC-3’ 5’-TCTTCTCATTCCCATACACC-3’	52
26	PKSTS D11	$(TC)_{9}$	5’-TAACACCTCTTTTCCCACAC-3’ 5’-AAGAAACAAATGGGTCACTG-3’	51
27	PKSTS D12	$(TA)_{7}$	5’-ACGTGACACTCCGAAATTAC-3’ 5’-ATCTACACCCAAAGATGACG-3’	52
28	PKSTS D13	$(AG)_{7}$	5’-TCGATGCCATTTCTTATTTT-3’ 5’-AATCCAAAATAGCATGCACT-3’	49
29	PKSTS D14	$(TC)_{7}$	5’-GATTCATGACCTCTGCTAGC-3’ 5’-AACAGTACCGCCAATAGTGT-3’	53
30	PKSTS D15	$(AT)_{7}$	5’-AGAAGGATATCAAAGCTCCC-3’ 5’-GATGTTCCAGAGACGTCAAT-3’	52
31	PKSTS T1	$(TTC)_{6}$	5’-TTTCTCGGTCACTTCAGTTT-3’ 5’-GGAAGACAAAGCACTGAGTC-3’	52
32	PKSTS T2	$(TTC)_{11}$	5’-GGTAATTTCCCCTTCTGTTT-3’ 5’-TATCCAAATCGAAAGGAAGA-3’	49
33	PKSTS T3	$(GAA)_{5}$	5’-GGAACATTCCTGAGAGCTTA-3’ 5’-TAAACAAATCTCCAACCAGG-3’	51
34	PKSTS T4	$(CTT)_{5}$	5’-CCATCTTGAGAAGTGATGGT-3’ 5’-GCATTCAGGTGAAGAAGAAG-3’	52
35	PKSTS T5	$(TGC)_{5}$	5’-GATTCTGACGTAATTGGAGC-3’ 5’-CTCGAAGAATCCAAACTCAC-3’	51
36	PKSTS T6	$(GAA)_{5}$	5’-GGCAGCTGAAGATATCAAAC-3’ 5’-GGTTTCCTTCTCCTCATTTT-3’	51
37	PKSTS T7	$(TCG)_{5}$	5’-TTCAAATCAAATTCAGGGAC-3’ 5’-AAATTCTCTCTCCCAACCTC-3’	50
38	PKSTS T8	$(ATA)_{6}$	5’-CTCATCTACCCGTTTTTCAG-3’ 5’-TAATGCCATTACTAGCAGCA-3’	51
39	PKSTS T9	$(AAG)_{5}$	5’-CAGTAGAGTCGTCAAGTGCA-3’ 5’-CCCTTTTCCAATAATCTCCT-3’	52
40	PKSTS T10	$(AGC)_{5}$	5’-TCTCAAGATCCGCAAATACT-3’ 5’-ATGTGGTTGTAGAAACGGAG-3’	52
41	PKSTS T11	$(GCA)_{6}$	5’-CTTTGGATAAGTTCCTGCTG-3’ 5’-TCCGAATCAATATTGGAGAC-3’	50
42	PKSTS T12	$(GCT)_{6}$	5’-CTTAGGAGCATGTCCTCATC-3’ 5’-CATCCCTAGAAATTCTCCCT-3’	52
43	PKSTS T13	$(ACC)_{6}$	5’-ATCACCACTTGTTCCACTTT-3’ 5’-ATAACACATCTGGTGGTGGT-3’	53
44	PKSTS T14	$(TGG)_{6}$	5’-AAAGCAATGCTCTGTCTCAT-3’ 5’-TACAGTTCATCCGGGAATAC-3’	52
45	PKSTS T15	$(GAT)_{17}$	5’-GAGATTCCTTCTCGGAAAAT-3’ 5’-TTTATCGAATCTTCTTTCGC-3’	49
46	PKSTS TT1	$(TGGG)_{3}$	5’-ATTAAATTGTTCCATGCCAC-3’ 3’-AATCCTTTGCAGACGACTTA-5’	50
47	PKSTS TT2	$(ACTC)_{3}$	5’-TACGCTAATGCAGTTCTTCA-3’ 3’ TACGCTAATGCAGTTCTTCA-5’	53
48	PKSTS TT3	$(ACTC)_{3}$	5’-TACGCTAATGCAGTTCTTCA-3’ 3’-CGTATTGGTACAACTACGCA5’	52
49	PKSTS TT4	$(TGGC)_{3}$	5’-TAAGCACAAGTGTCGTTACG3' 3’-TTCTTATTTGGGATGTGACC-5’	53
50	PKSTS TT5	$(CCCA)_{3}$	5’ AACCTGCTCAGGTTTACAAA 3’ 3’ TTGGGATATTCTACCAATCG 5’	53
51	PKSTS TT6	$(CCAA)_{3}$	5’ TATCAACATCATTCCACCCT3’ 3’GTGTCTTCTGAGCTGAGGAG5’	53
52	PKSTS TT7	$(GTTC)_{3}$	5’ CCAATGTTGCCTAGGTTATC3’ 3’GTCTCAATAGTGACCTTGGC5’	52
53	PKSTS TT8	$(AACA)_{3}$	5’ CTTCTTTAATTTCTTCGCCA3’ 3’CACAACTTCAAATGAAGCAA5’	49
54	PKSTS TT9	$(AACA)_{3}$	5’TCACACTTCCAAACACAAAA3’	52

			3’CATCTCTCAGCTCTTCGACT5’	
55	PKSTS TT10	$(TTGC)_3$	5’TGCCAATACTTGACTCACAA3’ 3’TTACAGTTCTGCTCGGTTTT5’	52
56	PKSTS TT11	$(AAGA)_3$	5’ATCACCTTACGGTTCAGATG3’ 3’ATAATGTTCGCAAAGGTGAC5’	52
57	PKSTS TT12	$(AAGA)_3$	5’ATCAGCAGTTGCCAGACTAT3’ 3’TATCTGCCAAAAACACATCA5’	54
58	PKSTS TT13	$(CCAA)_3$	5’CCTAAGAGAAGAACGAGCAA3’ 3’TTTTTCCTAGTTTTCCTGCA5’	51
59	PKSTS TT14	$(AGGG)_3$	5’AGGTTTTAGGATTTTAAGCCA3’ 3’GATTTTTCTTCCATTTGCTG5’	50
60	PKSTS TT15	$(TGGT)_3$	5’CTCATTATGCAGTTGGTGTG3’ 3’CAGCTAAGCATATGGAATCC5’	51
61	PKSTS P1	$(TTATT)_4$	5’-ACGAGTTTTCTGCAGATGAT-3’ 5’-AACCAAATTGAACACACACA-3’	51
62	PKSTS P2	$(AATCC)_3$	5’-AAAGTTGAAGAAGTCCGACA-3’ 5’-TCAGTCGCAGTTCAAGTATG-3’	52
63	PKSTS P3	$(CGTAT)_3$	5’-AAAGAAGAGCCGGACTATTT-3’ 5’-TTTGGAATATATTGGCAGCT-3’	50
64	PKSTS P4	$(CAAAA)_3$	5’-ATGGAAAGGCATCAGAATTA-3’ 5’-TATCTGTTCCTTGCCATTTT-3’	49
65	PKSTS P5	$(TTCGA)_3$	5’-GACGACGGAGAGGAGAAC-3’ 5’-CTGTTTGCAAACTCCAGATC-3’	53
66	PKSTS P6	$(TTTCA)_3$	5’-CGCTTAACATGTACTCCCTC-3’ 5’-CGGTCACTGTTTGAGACTTT-3’	53
67	PKSTS P7	$(CTGCA)_3$	5’-GAATGTCTCTTCGTATTCCG-3’ 5’-GCTGGACACAGGAACATTAT-3’	51
68	PKSTS P8	$(TTTCG)_3$	5’-CATCGGTACTTTTCCTAACG-3’ 5’-ACCAATGATCTCGATTATGC-3’	50
69	PKSTS P9	$(TGGTG)_4$	5’-TGCTTTCGACTAGCTTCTTC-3’ 5’-CGTCTATCAGAATAATCGGC-3’	51
70	PKSTS P10	$(GTGTT)_3$	5’-AAAAAGGTAAGAAATGCGG-3’ 5’-GATGGGACAAGAGATCTCAA-3’	50
71	PKSTS P11	$(TTATG)_3$	5’-GGTCTTGTTCTTGGGTTTTA-3’ 5’-AAAAATAGAACCTGGCAACA-3’	50
72	PKSTS P12	$(CCCAT)_3$	5’-GAACTTGAAGAATGCAAAGG-3’ 5’-GCCAGCTAAAAATGACCTTA-3’	50
73	PKSTS P13	$(ATCAC)_3$	5’-TTTTCCCAGGTAAAAATTGA-3’ 5’-AGCTGTGAGATCAGAGGATG-3’	50
74	PKSTS P14	$(ACACA)_3$	5’-TATAAAATCGCACCAGGTTT-3’ 5’-ACAATCCACAGCACTGAGA-3’	52
75	PKSTS P15	$(CAAGA)_3$	5’-AAGAGTGCTAGGACACCTGA-3’ 5’-GACGAAGTTTTCACTGCTTC-3’	53
76	PKSTS H1	$(TTTTTC)_3$	5’-AATGACCATTAAATTGTGCG-3’ 5’-TCTTTCTATTGTTTTTGCCC-3’	49
77	PKSTS H2	$(GAAAAA)_3$	5’-TCTTTCTATTGTTTTTGCCC-3’ 5’-AATGACCATTAAATTGTGCG-3’	49
78	PKSTS H3	$(GGTGAA)_3$	5’-CAAAGTCAACAAGAAGGGAG-3’ 5’-CTTGCTGATCTTCTTAGCGT-3’	52
79	PKSTS H4	$(GAAAGA)_3$	5’-CGGGACACCTTAATCATTTA-3’ 5’-CCTAGACACTCTAGGAGGGG-3’	52
80	PKSTS H5	$(CGGGAA)_8$	5’-AAGGTCTTCTTTACGCTCCT-3’ 5’-CTATCTCTTTCCCGTACCCT-3’	53
81	PKSTS H6	$(GTAGCA)_3$	5’-GAAGTTGAAGCTCTGGCTTA-3’ 5’-TCCTTGACCTTGCTTCTAAG-3’	52
82	PKSTS H7	$(AGATCG)_3$	5’-GCTGTCAAATGTTGACAGTG-3’ 5’-TAGCAATTATGTTGCTGACG-3’	51
83	PKSTS H8	$(CTCCAG)_4$	5’-GTTTTATTCGTGTTTACCGG-3’ 5’-TAACATACCGACCAGGATTC-3’	50

84	PKSTS H9	$(AGATGA)_3$	5’-CCGTCTGTAGACCATGCTAT-3’ 5’-CGTATCATCCTCTTCACCAT-3’	53
85	PKSTS H10	$(TCTGGC)_3$	5’-CTGACCAGCTAGCTTCTTGT-3’ 5’-GGAAGAGGAGAAGGTCAAGT-3’	54
86	PKSTS H11	$(TCGAGC)_3$	5’-TCGTCATACCTAGAGCCTTC-3’ 5’-TCTCACACAACACACACACA-3’51	54
87	PKSTS H12	$(AAATCC)_4$	5’-TTCATTTTGGCACATGAGTA-3’54 5’-TGCAAACACTCAAACTTTAACT-3’	49
88	PKSTS H13	$(GAACCT)_5$	5’-TTAGAGGAGGAAGCTAGGGT-3’ 5’-TAAGTCAGCTTCTCAGGCTC-3’	54
89	PKSTS H14	$(AGATGA)_3$	5’-TTTGTAGCTTGCAATGAATG-3’ 5’-GCAGAAATCGTATCGTCTTC-3’	50
90	PKSTS H15	$(GGTGGA)_3$	5’-GAATTCGCTTGACGAATTAC-3’ 5’-TTTGATTCCTTAAACAGGGA-3’	49
91	PKR25 M1	$(T)_{14}$	5’-TACTGGTCTTAGAACCCGAA-3’ 5’-ATGATTTCCAACGAAATCAG-3’	50
92	PKR25 M2	$(A)_{12}$	5’-CATAGATTCGATCGTGGTTT-3’ 5’-TCTTCTTCTTCGGACACATT-3’	51
93	PKR25 M3	$(A)_{14}$	5’-TAATCTAAAGGGAGTTCCCC-3’ 5’-AAACAATTTGAAAGAACCGA-3’	50
94	PKR25 M4	$(T)_{12}$	5’-CTGGCCTAACTAAACATTGG-3’ 5’-AAATCACACATTAACTCGGC-3’	51
95	PKR25 M5	$(T)_{12}$	5’-GTTGCGATATGGTGGTAGTT-3’ 5’-AAGTTTAGCAAAAGCATTGC-3’	51
96	PKR25 M6	$(A)_{18}$	5’-CCACAATCAAGTCATAACGA-3’ 5’-TTAGCTGCGTTATTTGCATA-3’	50
97	PKR25 M7	$(T)_{20}$	5’-GGGATTGCAAAACTGTTTA-3’ 5’-ATATGACGAACAATCCATTAT-3’	48
98	PKR25 M8	$(T)_{12}$	5’-AAAGAAGACAATCCCACTCA-3’ 5’-AAAAAGCCTAAACCTTTGCT-3’	51
99	PKR25 M9	$(A)_{18}$	5’-AAGACAACCGCAAATAAAGA-3’ 5’-TTTGTTCGTTCCTTCTGTTT-3’	50
100	PKR25 M10	$(A)_{12}$	5’-GTTGCTTACATCCCAATCAT-3’ 5’-CCACATGTTTATCCGGTAGT-3’	51
101	PKR25 M11	$(A)_{14}$	5’-AGGTGGAGTCGAAATGACTA-3’ 5’-GGATTCTCGGATTTGTAGAA-3’	51
102	PKR25 M12	$(C)_{12}$	5’-AAAACACAGTAAAACTCGGC-3’ 5’-ACCAGTGTTGGATAGGTTTG-3’	52
103	PKR25 M13	$(T)_{14}$	5’-CCCCAAGCAAAGTACAAGTA-3’ 5’-TATGAATCTGAACCACTGGG-3’	52
104	PKR25 M14	$(T)_{12}$	5’-AAAAGGCAAAAATCCTTTCT-3’ 5’-AAAGAAAGGCAAAAATCCTT-3’	48
105	PKR25 M15	$(A)_{12}$	5’-ATGACCTGATCAATTCGAAC-3’ 5’-CCTCCCCTACAGTATCATCA-3’	51
106	PKR25 D1	$(TA)_8$	5’-TAGGCGATTTATGATTTCGT-3’ 5’-CGTGAACATATTACGTGACG-3’	50
107	PKR25 D2	$(AT)_8$	5’-GAATGCTTGCACTTAGGATC-3’ 5’-CATGACGTGAACATATTTCG-3’	50
108	PKR25 D3	$(TA)_{12}$	5’-TTCGCAATCACAATAAACTG-3’ 5’-CCTATAAAAAGGGGAGGAAA-3’	49
109	PKR25 D4	$(AT)_7$	5’-TTTTTCAATGAAATATCGTGAA-3’ 5’-GGATTTGAACCGATGACTTA-3’	48
110	PKR25 D5	$(TA)_7$	5’-CAATAGCACTGGCACTCATA-3’ 5’-GCATTTTCTTTTGCAATCTC-3’	50
111	PKR25 D6	$(AT)_{10}$	5’-TGGTATTCATAACACTGGCA-3’ 5’-CTTCACAAATCGTGTCAATG-3’	50
112	PKR25 D7	$(AT)_7$	5’-GGCGTTACTCTACCACTGAG-3’ 5’-TTTTTCAATGAAATATCGTGAA-3’	51
113	PKR25 D8	$(TA)_9$	5’-TCAATATTGCACGTACACGT-3’	51

			5’-CGTGAATCAGATTTGTGTTG-3’	
114	PKR25 D9	$(AT)_{10}$	5’-TGTACAAATCCAATGAACCA-3’ 5’-CTACTTCCCAAATATTCCCC-3’	50
115	PKR25 D10	$(AC)_{7}$	5’-CAAATTTTGACTACCCAAGC-3’ 5’-TTTTCTTTTCTTGTTTTCGC-3’	49
116	PKR25 D11	$(AT)_{10}$	5’-GGTAGGGTACAGGTATGCAA-3’ 5’-AAAAATGCGACAGAGAGATG-3’	52
117	PKR25 D12	$(AT)_{8}$	5’-TAAATCGTCCCCAAAGTAGA-3’ 5’-TTCGATTCTATGGAGCATCT-3’	50
118	PKR25 D13	$(AT)_{8}$	5’-TTTAGAGGATTTGGTGCAAT-3’ 5’-TTGGGTAAGAGTTGCAAGTT-3’	51
119	PKR25 D14	$(TA)_{10}$	5’-AGAAGGAGTGCAAGCTTATG-3’ 5’-ATAAGAGAAGGAGGTGGAGG-3’	53
120	PKR25 D15	$(GA)_{8}$	5’-TATCTCAGTGCCCAACTTTT-3’ 5’-GTGACATTTTGGCTTTTCTC-3’	51
121	PKR25 T1	$(AAT)_{5}$	5’-CAAATATTGGGACGATATGC-3’ 5’-CCCGTCAAGTCTCTCTACAT-3’	51
122	PKR25 T2	$(AAT)_{5}$	5’-ATATTGGGACAATACGATGG-3’ 5’-CGTCCCAATATTTGAATCAT-3’	49
123	PKR25 T3	$(TTA)_{5}$	5’-TTGAAGGTACGAATCGAGTT-3’ 5’-TAAAACCCGGAGACGTAGTA-3’	52
124	PKR25 T4	$(AAT)_{5}$	5’-ATGATTCAAATATTGGGACG-3’ 5’-CCCCATCAAGTCTCTCTACA-3’	51
125	PKR25 T5	$(GAA)_{5}$	5’-CGAGAGACTAACCCTGAGTG-3’ 5’-AATGGACGAATTAATTGTGG-3’	51
126	PKR25 T6	$(CTC)_{5}$	5’-GATTACGACGAAGAATCAGC-3’ 5’-ACATTTTGTAGATGAACCGG-3’	51
127	PKR25 T7	$(GAG)_{5}$	5’-GTTTACCACCTGATGTGACA-3’ 5’-TCAATGTGTATTGCGAGAAA-3’	51
128	PKR25 T8	$(TTC)_{5}$	5’-AATGGACGAATTAATTGTGG-3’ 5’-CGAGAGACTAACCCTGAGTG-3’	51
129	PKR25 T9	$(AAG)_{5}$	5’-TGTTAACAGATGGTGGTGAA-3’ 5’-CCTTCATCTCAGTTTCCAAG-3’	51
130	PKR25 T10	$(TCT)_{5}$	5’-TTCCAGTTTCCAGGTTTTTA-3’ 5’-AGGATTTCTAGAGGTACCCG-3’	51
131	PKR25 T11	$(ATG)_{5}$	5’-TGTAGAGGAGGTTCGGTCTA-3’ 5’-GTATGGGTGCCAGTGTTAAT-3’	53
132	PKR25 T12	$(TCT)_{5}$	5’-TCCAGTCTTCCAGGTTTTTA-3’ 5’-GAAGGATTTCTAGAGGTGCC-3’	52
133	PKR25 T13	$(TCT)_{5}$	5’-GCTTCACAAGTCTTCGAATC-3’ 5’-TTTTCTTCTTAGAAGGCCCT-3’	51
134	PKR25 T14	$(TCT)_{16}$	5’-CGAGCTTCAATTTCATTTCT-3’ 5’-CCTAGTTCGAAGGGGTATTT-3’	50
135	PKR25 T15	$(ATT)_{15}$	5’-CTAGAACCGTGGTCAGAAAG-3’ 5’-GACAGATTTCATCTGCTTCC-3’	52
136	PKR25 P1	$(TAAAA)_{4}$	5’-AAAATGAACTGCAAAAACCT-3’ 5’-GGTCCAGTGGTTCAGATTTA-3’	50
137	PKR25 P2	$(GTTTA)_{3}$	5’-TGCTTGTTTAGTTTAGGTTGC-3’ 5’-AATTCAAACACCGAGCTAAA-3’	51
138	PKR25 P3	$(GTTTA)_{4}$	5’-TTTATCTTTTTGCATCCTTTTT-3’ 5’-GTTGCCTAATCTCATCAAGC-3’	49
139	PKR25 P4	$(TAAAC)_{4}$	5’-AATTCAAACACCGAGCTAAA-3’ 5’-TGCTTGTTTAGTTTAGGTTGC-3’	51
140	PKR25 P5	$(TAAAC)_{4}$	5’-AACCAAACAATTCAAACACC-3’ 5’-TTTGTTTAATCTTTTTCCTGTG-3’	48
141	PKR25 P6	$(TTTTA)_{4}$	5’-AACCGCAGACAAAATAAAAA-3’ 5’-GGGACAAGCAAATGTTTAAG-3’	49
142	PKR25 P7	$(TTTTA)_{4}$	5’-GGCACAATCTAATGGTCATT-3’ 5’-TTGAGGACAAGCAAAGATTT-3’	50

143	PKR25 P8	$(TTTTA)_5$	5’-TTAAAGAAAAACGGACTTGG-3’ 5’-CTAGACGGCTCATTCCTATG-3’	50
144	PKR25 P9	$(TTTTA)_4$	5’-AAGTACGGACCAAACAAAAA-3’ 5’-TTGAGGACAAGCAAAGATTT-3’	50
145	PKR25 P10	$(AAAAC)_3$	5’-GGCCTAGCAATCATATCAAG-3’ 5’-GTTGCGATATGGTGGTAGTT-3’	52
146	PKR25 P11	$(AGTTT)_3$	5’-TCTTGATGGATGATGAGGAT-3’ 5’-AATTCCACATACAAGGTTGG-3’	50
147	PKR25 P12	$(AGAAA)_3$	5’-TGGGAATGATCGAAATAAAC-3’ 5’-TCCTGATATCCACTTCTGCT-3’	50
148	PKR25 P13	$(TAAAA)_4$	5’-ACTCCGGGAAATTAAATTTC-3’ 5’-CTCCGCTAGTCTAGTGGAAA-3’	51
149	PKR25 P14	$(AAAAT)_3$	5’-GAATCGACCATTACAGAAGC-3’ 5’-AGACTCCGCTATTCATCGTA-3’	52
150	PKR25 P15	$(AAAAG)_4$	5’-CAACTAAGATCGAATTTGGG-3’ 5’-TCTGAATCTCAACCTCTGCT-3’	51
151	PKR25 TT1	$(AAAC)_3$	5’-TCCTTTCCTTGAACTTGAAA-3’ 5’-ATCGTTGTTGTTTCAAGGTC-3’	50
152	PKR25 TT2	$(GTTT)_3$	5’-ATCGTTGTTGTTTCAAGGTC-3’ 5’-TCCTTTCCTTGAACTTGAAA-3’	50
153	PKR25 TT3	$(CTTT)_3$	5’-ACCGTTCATTTTTAACGGTA-3’ 5’-AAAGGCGTGACATATTTAGG-3’	50
154	PKR25 TT4	$(TTTA)_4$	5’-TTTTTCTTTTCTTTCTTCGC-3’ 5’-ATAAAAAGGTGCAAACCAAA-3’	48
155	PKR25 TT5	$(TTGA)_3$	5’-CTGTAGTTGTGTTGCGTGTT-3’ 5’-GTAAAACTAGGCACGCAATC-3’	53
156	PKR25 TT6	$(CTCA)_3$	5’-GCCCATATCATGCTCATATT-3’ 5’-TGAGCCTTTTAAGCCATATT-3’	50
157	PKR25 TT7	$(CCCA)_3$	5’-AATCCTTTGCAGACGACTTA-3’ 5’-ATTAAATTGTTCCATGCCAC-3’	50
158	PKR25 TT8	$(TGGG)_3$	5’-ATTAAATTGTTCCATGCCAC-3’ 5’-AATCCTTTGCAGACGACTTA-3’	50
159	PKR25 TT9	$(AAAT)_4$	5’-AAAATTGAAAACAAGCGAAG-3’ 5’-TAAAATGTGGAGGAGAATGG-3’	49
160	PKR25 TT10	$(AAAG)_3$	5’-CTTATAATGCGGGCTGTAAC-3’ 5’-CCATGAGTTTGAGCAAAAAT-3’	50
161	PKR25 TT11	$(TTGA)_3$	5’-CACCTTTTTATTTTCGGTTG-3’ 5’-AAAAACAAACACATGCAACA-3’	49
162	PKR25 TT12	$(TCAA)_3$	5’-AATTGACAAACACATGAGCA-3’ 5’-GCGTGTTTGCTCTTATCTTT-3’	51
163	PKR25 TT13	$(AAAC)_4$	5’-CAAACCTATCCAACACTGGT-3’ 5’-TCTGAGGAGAATGGATCATC-3’	52
164	PKR25 TT14	$(AAAT)_3$	5’-GGCACAATCTAATGGTCATT-3’ 5’-AAGAAAAACGAACTTGGTCC-3’	51
165	PKR25 TT15	$(AAAC)_3$	5’-TGCCTACAGATACACAATTCA-3’ 5’-ATATGGTTGAATCGCGTATC-3’	51
166	PKR25 H1	$(TCTTTT)_3$	5’-TAAAATGTGGAGGAGAATGG-3’ 5’-AAAATTGAAAACAAGCGAAG-3’	54.6
167	PKR25 H2	$(AAAAAG)_3$	5’-ACCGGACATTTATTCTGAAA-3’ 5’-AAAGAAGACAATCCCACTCA-3’	50
168	PKR25 H3	$(AAAAGA)_3$	5’-AAAATTGAAAACAAGCGAAG-3’ 5’-TAAAATGTGGAGGAGAATGG-3’	49
169	PKR25 H4	$(AAAAAT)_3$	5’-ATACGTAAAACTCGGCCATA-3’ 5’-AGACTTTCCAAACACACCAC-3’	52
170	PKR25 H5	$(AAAAAT)_3$	5’-TTAAAGAAAAACGGACTTGG-3’ 5’-CTAGACGGCTCATTCCTATG-3’	50
171	PKR25 H6	$(AAAAAT)_3$	5’-ATACGTAAAACTCGGCCATA-3’ 5’-GACTTTCCAAAACACACCAC-3’	52
172	PKR25 H7	$(AAAAAT)_3$	5’-GTGTCGACGTCTCCTACAAT-3’	54

			5’-TTACTTGTCGACCGTGTACA-3’	
173	PKR25 H8	$(TTTCTT)_3$	5’-AGAGGCCGTAAGAGAGTTCT-3’ 5’-CTCCAATTCAACCATTGATT-3’	52
174	PKR25 H9	$(AAAAAG)_3$	5’-CTCCAATTCAACCATTGATT-3’ 5’-AGAGGCGGTAAGAGAGTTCT-3’	52
175	PKR25 H10	$(ATTTTT)_3$	5’-TTGGATGTCGATAGACTTCC-3’ 5’-TGGGTTTATCTCAACTGCTT-3’	51
176	PKR25 H11	$(TAAAAA)_3$	5’-TCAGCAAACGGATTTTAGAT-3’ 5’-CCCAAAAAGTCTGAAACAAC-3’	50
177	PKR25 H12	$(TAAAAA)_3$	5’-TCCTTAGCCTGGAGTGATAA-3’ 5’-AAAATAAAACTCTAGCGGGG-3’	51
178	PKR25 H13	$(TCGTCA)_4$	5’-GATTACGACGAAGAATCAGC-3’ 5’-ACATTTTGTAGATGAACCGG-3’	51
179	PKR25 H14	$(AAAAAT)_3$	5’-GGAGGAGCTAAGGATAAAGG-3’ 5’-TCAACCAAAACAACCTAAACT-3’	51
180	PKR25 H15	$(AAAAAC)_3$	5’-GCATTTGCTCTCTTATGTCC-3’ 5’-ATGGGTTACAAGAAGTGGTG-3’	52
181	PKS15 M1	$(T)_{48}$	5’-TCAATAGACTGCTAAAGCCC-3’ 5’-TCGTCTTTTTGGTCTCATTT-3’	53
182	PKS15 M2	$(T)_{12}$	5’-AATTCAGAAAATTGGTTCGA-3’ 5’-AGGTCCCCTCAGATCTAAAC-3’	51
183	PKS15 M3	$(T)_{18}$	5’-ACGACAAAAAGAACTCCTGA-3’ 5’-CTTTCAAGATTTATCGACCG-3’	52
184	PKS15 M4	$(A)_{12}$	5’-CCAACTATGTCAGGAAAAGG-3’ 5’-CGCGATTGATTTCTAAGATC-3’	50
185	PKS15 M5	$(T)_{16}$	5’-GATTATGAACCAAAAACCGA-3’ 5’-ATTTATCCGCGATCTAGACA-3’	52
186	PKS15 M6	$(T)_{12}$	5’-GAGATTGAAATACCGATCCA-3’ 5’-AAGGAGACATTTTTCCTTCC-3’	50
187	PKS15 M7	$(T)_{12}$	5’-AAACCCACAAATGTACAAGG-3’ 5’-TTGGTCTATGAGCTGGAATT-3’	53
188	PKS15 M8	$(A)_{12}$	5’-GCGACAAGTACGAGTAGCTT-3’ 5’-GAGATTATAGGGTTGGGGTT-3’	55
189	PKS15 M9	$(T)_{12}$	5’-CGGTAATATCTTAGCGGATG-3’ 5’-TGGACGAAAGGAGAATTTTA-3’	52
190	PKS15 M10	$(T)_{12}$	5’-AGTTAAGCTAAATTCGCGTG-3’ 5’-CTCCATCAATATTTTGGGAA-3’	51
191	PKS15 M11	$(A)_{12}$	5’-GCGACAAGTACGAGTAGCTT-3’ 5’-GAGATTATAGGGTTGGGGTT-3’	55
192	PKS15 M12	$(A)_{12}$	5’-AGGAGGAGCTTAATTTCGTT-3’ 5’-GAACTTGGTGTGAGGAAAAC-3’	51
193	PKS15 M13	$(G)_{16}$	5’-TGGCGTAAGCTTAAGACAAT-3’ 5’-GGCTCAGACATATGGTCATT-3’	52
194	PKS15 M14	$(A)_{12}$	5’-CACCCACTTTCTGAGATCAT-3’ 5’-CCTTTGAAAACATTGTAGCC-3’	49
195	PKS15 M15	$(T)_{12}$	5’-ATCACTTATTTTGTCGCGTT-3’ 5’-CGCCCAAAAAGAAAATTATA-3’	50
196	PKS15 D1	$(TG)_9$	5’-ACTGTGGCCTAGTTGAAGAA-3’ 5’-ATGAAACGTCATCTCGAATC-3’	52
197	PKS15 D2	$(AC)_{10}$	5’-TTAGCATCTGTCTTCGGAAC-3’ 5’-TAAAGAGATTTTGAAGCCCA-3’	53
198	PKS15 D3	$(TC)_{10}$	5’-ATGACAGATTGGGTTTCTTG-3 5’-GAAATGATGTCCCGATAAGA-3	50
199	PKS15 D4	$(AC)_{12}$	5’-GAAACAATTTGGATTTAGCG-3 5’-CAACATCACCACATTCAAAC-3’	49
200	PKS15 D5	$(AG)_7$	5’-AAAAGGTCTCCTTCATCCTC-3’ 5’-TCTTTTGGTGGTCTTATGCT-3’	52
201	PKS15 D6	$(TC)_8$	5’-CAGCCAAGAAAGATCTATCG-3’ 5’-CCAGGTGATAAATCCACAGT-3’	51

202	PKS15 D7	$(AG)_9$	5’-CTCTCGCTTTCATTCAAAAT-3’ 5’-CAAGTTGAAGGCTAACCAAG-3’	50
203	PKS15 D8	$(TC)_{10}$	5’-TCCAACAACTCAGAGGATTC-3’ 5’-AGGGATACATAAGTTGGGGT-3’	50
204	PKS15 D9	$(TA)_9$	5’-GTTTGCCCTAAATAAGGGTT-3’ 5’GAGGAAAGTAGCATGTGGAC-3’	52
205	PKS15 D11	$(AG)_{13}$	5’-AATATACACTCCATGTCCGG-3’ 5’-CCTACCTTCCCTGGTATTTT-3’	51
206	PKS15 D13	$(CT)_7$	5’-AAATCGAACAATAACGCTGT-3’ 5’-GTGGTTACAGGTGGAGTTGT-3’	52
207	PKS15 D14	$(CT)_{14}$	5’-ACAGGTTCTCTCACGAACAC-3’ 5’-CAGCAATGAATCTCAAATGA-3’	51
208	PKS15 D15	$(GA)_7$	5’-TCTGTACTTGGCCTTTGTCT-3’ 5’-CCAAAAATAACTCTCAACGG-3’	49
209	PKS15 T2	$(GAA)_6$	5’-CGTGACGTTTTAAGCATACA-3’ 5’ACAGAAATGGTCTTTTGTTGA-3’	51
210	PKS15 T3	$(ATT)_6$	5’-TTTTTCAAATTCTAACCCCA-3’ 5’-ATTTTCAAATCAAACGGCTA-3’	50
211	PKS15 T5	$(TGG)_6$	5’-AAAGCAATGCTCTGTCTCAT-3’ 5’-TACAGTTCATCCGGGAATAC-3’	54
212	PKS15 T7	$(GGT)_5$	5’-TCATCATGACGTTCTTTTGA-3’ 5’-AACATGACATTGTCAAGCAA-3’	48
213	PKS15 T8	$(TCT)_5$	5’-GGTTTCCTTCTCCTCATTTT-3’ 5’-GGCAGCTGAAGATATCAAAC-3’	51
214	PKS15 T9	$(CTT)_5$	5’-CCATCTTGAGAAGTGATGGT-3’ 5’-GCATTCAGGTGAAGAAGAAG-3’	54
215	PKS15 T10	$(AAG)_5$	5’-ACACAACATTCAAAGATGCA-3’ 5’-GCTTCTGCTGATTTGACTCT-3’	50
216	PKS15 T11	$(TCT)_7$	5’-CCCCAATAAATAAACAGTCG-3’ 5’-AATCTCTAGAAGCTCCCTTGA-3’	49
217	PKS15 T12	$(TCT)_5$	5’-GGTTTGTTTATCTTTGCAGG-3’ 5’-AAACCGCAATCTCAATCTAA-3’	52
218	PKS15 T13	$(TCT)_5$	5’-CCCCAATAAATAAACAGTCG-3’ 5’-AATCTCTAGAAGCTCCCTTGA-3’	53
219	PKS15 T14	$(AAG)_5$	5’-CAGTAGAGTCGTCAAGTGCA-3’ 5’-CCCTTTTCCAATAATCTCCT-3’	52
220	PKS15 T15	$(ATA)_5$	5’-CTCATCTACCCGTTTTTCAG-3’ 5’-TAATGCCATTACTAGCAGCA-3’	53
221	PKS15 TT1	$(CAAT)_3$	5’-GTGGATTGATGACGAGAGAT-3’ 5’-AATTCTTCTCTTTACCTTACCAAA-3’	51
222	PKS15 TT2	$(ATTA)_3$	5’-TTTCTGGTGGGTATCCTTAA-3’ 5’-TCGACTATATCCGCATTTTT-3’	52
223	PKS15 TT3	$(TGGG)_3$	5’-ATTAAATTGTTCCATGCCAC-3’ 5’-AATCCTTTGCAGACGACTTA-3’	52
224	PKS15 TT4	$(TGTT)_3$	5’-GAATGAAGCCAAGTCTGAAG-3’ 5’-TGGCTTCTTTAATTTCTTCG-3’	52
225	PKS15 TT5	$(GAAA)_3$	5’-GAAAGCGTGTCATCTTTCTC-3’ 5’-AAGTGGAAAAATGCCATTTA-3’	52
226	PKS15 TT6	$(TTTC)_3$	5’-CGAAATTCAGGAGACAGAAG-3’ 5’-TTGAGGTTTGTTCCTTGACT-3’	53
227	PKS15 TT7	$(ATCT)_3$	5’-CATTTAACCGTAGTGCATG-3’ 5’-CCGGTCCACTACCATAAATA-3’	53
228	PKS15 TT11	$(TTTC)_3$	5’-AATCTTCGGACCCTTTAGTC-3’ 5’-CCAAGAAGAAACAGGAAATG-3’	52
229	PKS15 TT12	$(TTCC)_3$	5’-GTGCTGCAAGCAATATATCA-3’ 5’-GCTGGAATATGATAGGAACG-3’	53
230	PKS15 TT14	$(TATG)_3$	5’-GCTTAACAGCACCCAACTAC-3’ 5’-TCTACACACCATGCACTCTT-3’	56
231	PKS15 TT15	$(ACGA)_3$	5’-GCCGTCAGTTCTTAAAGATG-3’	50

			5’-AAACCAAAAATTAACTGGCA-3’	
232	PKS15 P1	$(ATGTG)_3$	5’-TGAATTTGTGTTTGGATTGA-3’ 5’-GTCAAGTGTGTTTGTTGACG-3’	52
233	PKS15 P3	$(AATGA)_3$	TAGTTCCCCCACATATGTTC AAGGCAGATCGTTCAGTAAA	54
234	PKS15 P4	$(AAAAT)_3$	5’-AAGCGCAAAACAATTACAAC-3’ 5’-GCCTCGAAAAATATTGGTAA-3’	51
235	PKS15 P5	$(TACAA)_3$	5’-GAGAAATTCTGCCAATTGAC-3’ 5’-CAAAGATTCTAAGCGGAAGA-3’	52
236	PKS15 P6	$(CAGGT)_3$	5’-GGATGAAAATACGAATCGAA-3’ 5’-TTGGTGGTATTAGGATCAGG-3’	52
237	PKS15 P7	$(TTTTC)_3$	5’-TGAGGGGTTACTTGACAGAC-3’ 5’-TTTCCTTGCTCTTCTAGCAC-3’	55
238	PKS15 P8	$(AACTC)_3$	5’-CAAGGTCAAAGAGTCTCACC-3’ 5’-TGCAACTGATAAGTGGTCTG-3’	51
239	PKS15 P9	$(TATA)_3$	5’-AGGACGTGGAATAATGACAC-3’ 5’-GCAACCATATCTAAGACCCA-3’	50
240	PKS15 P10	$(ACAAA)_3$	5’-TTGTCTTTCGAGGATAAGGA-3’ 5’-CAACTTTGCCTTTTTCGTAC-3’	50
241	PKS15 P11	$(GATTT)_3$	5’- TCATGGTTTGGCTTCTTAGT -3’ 5’- TCTATCACTGCGTCTTGTTG -3’	54
242	PKS15 P12	$(TAAAA)_3$	5’-ATGTCGAGCAAAATTCAGTT-3’ 5’-CGATGTGCTGTTTTAGTTGA-3’	53
243	PKS15 P13	$(TTTCC)_3$	5’-ACAGTTCTTTTCCCCTCTTC-3’ 5’-ATGACGAGTATCCTTCATGG-3’	54
244	PKS15 P14	$(GAAAA)_3$	5’-GATTGGTGGTGAACGATTAT-3’ 5’-CACCACAGACTAAAACACCA-3’	53
245	PKS15 P15	$(TTTCC)_3$	5’-CCTAGTAATGGTTGCAAACC-3’ 5’-GAAGAAGAGAGGGAACAGGT-3’	50
246	PKS25 M1	$(T)_{12}$	5’-CCGGAAATACTAGGCCTACT-3’ 5’-CGATTGTCATAGGTATCTTTCA-3’	52
247	PKS25 M2	$(A)_{12}$	5’-CGTAAGACAAAATTCGTTTTT-3’ 5’-TAATCGTACCGCGTAATCTT-3’	49
248	PKS25 M3	$(T)_{14}$	5’-ACGACAAAAAGAACTCCTGA-3’ 5’-CTTTCAAGATTTATCGACCG-3’	50
249	PKS25 M4	$(A)_{12}$	5’-TGGACGAAAGGAGAATTTTA-3’ 5’-CGGTAATATCTTAGCGGATG-3’	50
250	PKS25 M5	$(T)_{12}$	5’-ATAAGTTTGAGCCTTCCCTC-3’ 5’-ATGTCAAGATCCCACACAAT-3’	52
251	PKS25 M6	$(A)_{14}$	5’-GGCAAGTCTACATGAGAAGC-3’ 5’-ATGGATTCAATGTCAAATCC-3’	51
252	PKS25 M7	$(A)_{14}$	5’-CCATCAAGCATGCATAATAA-3’ 5’-CTTTTCTCACTCCCAGAATG-3’	50
253	PKS25 M8	$(A)_{12}$	5’-GCTAATGAGAAACCAATTCG-3’ 5’-AAATTTTAGGGGACGAATTC-3’	49
254	PKS25 M9	$(T)_{12}$	5’-TCCTCAGCTTCACTTCAAGT-3’ 5’-AGAAGCACGCTAGAAAACAC-3’	53
255	PKS25 M10	$(T)_{12}$	5’-AGAGAACAGCCACAAAGGTA-3’ 5’-CACATATGAGAAGCCAGTGA-3’	53
256	PKS25 M11	$(T)_{14}$	5’-ATGATCAATTCCTGAACTGG-3’ 5’-CCAGTTATCCATTCGTACGT-3’	51
257	PKS25 M12	$(T)_{12}$	5’-TTGGTGAGGTCCTTTACAAG-3’ 5’-TAATATGCAGCTGCTTTTCA-3’	51
258	PKS25 M13	$(A)_{12}$	5’-GGCTTAGGATTAAGCATGTG-3’ 5’-GTAACGTAACGGATATCCCA-3’	51
259	PKS25 M14	$(T)_{12}$	5’-CAATTCACTCCAATGGAGTT-3’ 5’-AGCAAGAGCAGCATTAACTC-3’	52
260	PKS25 M15	$(A)_{12}$	5’-CTTGTGACGTAAGCAACAGA-3’ 5’-ATATGAGCATCATCCTCGTC-3’	52

261	PKS25 D2	$(TA)_{12}$	5’-AAATAGTGGAGCCTGATCCT-3’ 5’-GCAAACAAGGAATGTCGTAT-3’	52
262	PKS25 D3	$(TC)_{7}$	5’-GAACTTAAGTGAGCGGAAAA-3’ 5’-AAATTGAGAAATATGGCTGC-3’	50
263	PKS25 D4	$(CT)_{14}$	5’-ACAGGTTCTCTCACGAACAC-3’ 5’-CAGCAATGAATCTCAAATGA-3’	51
264	PKS25 D7	$(TC)_{10}$	5’-CCTTCTCACCTAATTCCCTT-3’ 5’-ACGGAAAGGAGATTGAATCT-3’	51
265	PKS25 D8	$(AG)_{10}$	5’-CCCAAGGGAAGATCTAAATT-3’ 5’-CCAAGGTCTTTGAACTTCAC-3’	50
266	PKS25 D9	$(CT)_{10}$	5’-TCTTCTCCTTCATCTTCAACA-3’ 5’-AGAAGGGAGTTTGAGGGTT-3’	52
267	PKS25 D10	$(GT)_{7}$	5’-CAACTTTCACGACCGTTAAT-3’ 5’-GAAGCTCTTGCAGAAAAAGA-3’	51
268	PKS25 D13	$(AT)_{7}$	5’-GTAAACTGTCAAACAAGCCC-3’ 5’-GTTTTTCAAAAGCATTTTGG-3’	49
269	PKS25 D14	$(AT)_{7}$	5’-ACACAAGGTATGCAAGCTTT-3’ 5’-GTTCATTGATCCATGACATG-3’	51
270	PKS25 D15	$(CT)_{10}$	5’-TTCTCGAGGCATTAATTTGT-3’ 5’-CTTTCCACGAAAGAGCTAAA-3’	50
271	PKS25 T1	$(AGC)_{5}$	5’-GAAGTTGAAGCTCTGGCTTA-3’ 5’-TCCTTGACCTTGCTTCTAAG-3’	52
272	PKS25 T2	$(AGC)_{5}$	5’-TCCTCATCGAATTCATCTTC-3’ 5’-ATGCTGAAGATACGCAAAAT-3’	50
273	PKS25 T3	$(TCA)_{9}$	5’-CATCATCACCATCATCTTCA-3’ 5’-GCTAATGAAAATTCATTGGC-3’	49
274	PKS25 T4	$(AAG)_{5}$	5’-AGCAAATGAAGAAGCAGAAG-3’ 5’-CTCATGGTCTCCACAAGATT-3’	51
275	PKS25 T5	$(TGA)_{7}$	5’-ATGCTTCAACTGCTGATCTT-3’ 5’-TCAACCATGTCGTCGTTATA-3’	52
276	PKS25 T6	$(TGA)_{7}$	5’-CTTCTACTGCTGATCTTGGG-3’ 5’-TCAACCATGTCGTCGTTATA-3’	52
277	PKS25 T7	$(AGA)_{5}$	5’-GAAGTAGATGTGGACAAGCC-3’ 5’-TTCTCGGTCACTTCAGTTTT-3’	52
278	PKS25 T9	$(TAT)_{5}$	5’-AAGAGGAAGGTGAAAAGACC-3’ 5’-GGCACATTCTTGAGATCTGT-3’	52
279	PKS25 T10	$(ATG)_{6}$	5’-GAGGAGGATGAAGATGATGA-3’ 5’-TTCATCCAATAATCAAACCC-3’	49
280	PKS25 T11	$(TAA)_{12}$	5’-TTTCCAGACCATAATTACGG-3’ 5’-TGAAGCCTTTAGTTGTTTGG-3’	50
281	PKS25 T12	$(TTC)_{5}$	5’-AATTTTCCAAACGTCTCAAA-3’ 5’-CAAAGAAACGGTTCAAAGTC-3’	49
282	PKS25 T13	$(GAT)_{5}$	5’-ATGCTGTTGGATTTTTACGT-3’ 5’-ATCTTGAAAGCCTTCAATCA-3’	50
283	PKS25 T14	$(GTG)_{5}$	5’-AAAGAGTGACGAAGGTGCT-3’ 5’-CTCTTGCTGCTACTGTTTCC-3’	54
284	PKSR M1	$(T)_{14}$	5’-ACAGGGAGAAATGTGTTTTG-3’ 5’-GACAAGGTGAAAGGATGTGT-3’	52
285	PKSR M3	$(A)_{12}$	5’-GGTCACCTTCCTAATTCACA-3’ 5’-AACGTTGAAGTTGAAAAGGA-3’	51
286	PKSR M4	$(A)_{12}$	5’-ATCAGCAGCATAACAAGGTT-3’ 5’-TGGAATATTTGTTTGGAAGG-3’	50
287	PKSR M5	$(T)_{12}$	5’-ACGGAAAATTCAAAATGATG-3’ 5’-GATCGAGTCAAATCCACATT-3’	49
288	PKSR M6	$(A)_{12}$	5’-ATCATGGGTTTTCAAAGTTG-3’ 5’-GTGAAATGATCCCGTCTATG-3’	50
289	PKSR M7	$(A)_{14}$	5’-AAAAAGGAATAATTGGTTCAAG-3’ 5’-TGCTGTTCTAAGAGAAAGGG-3’	50
290	PKSR M8	$(A)_{14}$	5’-CGATACTGTAAAATCCAGGG-3’	52

			5’-TCAAGTAGCTCTTGCTCCTC-3’	
291	PKSR M9	$(A)_{18}$	5’-ACAAATAATTCAGGCTCCAA-3’ 5’-ATGAGCGGAGTCGTATTAAA-3’	50
292	PKSR M10	$(A)_{12}$	5’-GCTTATTTTCCAACAATCGT-3’ 5’-GTTCCGTCAAGAGTTCAATC-3’	50
293	PKSR M11	$(T)_{12}$	5’-TGTAAGCATACAAATGCTGC-3’ 5’-GGTAATTATTGGGTTAGGGC-3’	51
294	PKSR M12	$(T)_{16}$	5’-CCAGCTTGGTAACAAATTTC-3’ 5’-GCTGAACTAATTGCTCAGCT-3’	51
295	PKSR M14	$(A)_{14}$	5’-TAGATGTAACACGCATTTGC-3’ 5’-GAAGAGGATCTGAGGTTTCC-3’	52
296	PKSR M15	$(A)_{16}$	5’-TCAACTCCTGAAGATCAGCT-3’ 5’-GGTAGACTCGTGCACCATAT-3’	54
297	PKSR D2	$(AG)_{7}$	5’-CCTGTTTATCTTCCAATGGA-3’ 5’-CCTACAAAAGGTACAGTGCC-3’	51
298	PKSR D3	$(TC)_{11}$	5’-TCTGCGGTTGAAATTCTACT-3’ 5’-ACTAAAATTTGTGCCTTGGA-3’	51
299	PKSR D4	$(AG)_{8}$	5’-CATCATTGTATTGATCGCAG-3’ 5’-CCATACACCACCCAAATAAT-3’	50
300	PKSR D5	$(AT)_{8}$	5’-CAAATTTCGAAGTACTTGGG-3’ 5’-CAAAAGCTCCCATCTAAATG-3’	52
301	PKSR D6	$(AT)_{8}$	5’-AAATGTAATCGGACAAGTGG-3’ 5’-ACTCAAGAACAAGTCGGAAA-3’	51
302	PKSR D8	$(AT)_{7}$	5’-CTCAATTTGACAGCACAGTG-3 5’-ACATCTCATTAAGCGCAATT-3	51
303	PKSR D10	$(AC)_{8}$	5’-TGCTAGAGTGATTGCAGATG-3’ 5’-TCGATTTTACAACATCCACA-3’	51
304	PKSR D11	$(AT)_{10}$	5’-TTTTTGTTACAAGCGGCTAT-3’ 5’-AACCAAATGAAACTGCAAAT-3’	50
305	PKSR D12	$(TC)_{7}$	5’-TAATTCGCATAACTGCACTG-3’ 5’-GTGGAACTTCTTGCGTAGAC-3’	52
306	PKSR D13	$(AT)_{8}$	5’-AGCAAAGTTGGAAATTCAAA-3’ 5’-TTCCGATGGATTATGTGAAT-3’	49
307	PKSR D14	$(AT)_{7}$	5’-CTTTCGCAGTAGCCATTTAT-3’ 5’-AGCGTTACACAAGAAGAAGG-3’	52
308	PKSR D15	$(TA)_{12}$	5’-ATCTGTAACCCTCTGCACTG-3’ 5’-CCCTTTTGGATATTTTCACA-3’	51
309	PKSR T3	$(TGA)_{5}$	5’-CTCACATCTGGGTTCAGTTT-3’ 5’- TGTACGGTAGAACATCTCCC -3’	53
310	PKSR T4	$(CAG)_{6}$	5’-CATCCCTAGAAATTCTCCCT-3’ 5’-CTTAGGAGCATGTCCTCATC-3’	52
311	PKSR T5	$(ATC)_{5}$	5’-AACCTTAATCTGCAGCATGT-3’ 5’-TCAAAGTCATGCAACTGGTA-3’	52
312	PKSR T6	$(ACC)_{5}$	5’-AGGTGCTCCTGCTAATAATG-3’ 5’-CGAAAACGGTATAGGTGAAG-3’	51
313	PKSR T7	$(CAT)_{5}$	5’-ATTAGGCACATCAACCAGTC-3’ 5’-TTTGCAATAATTCAGCAATG-3’	50
314	PKSR T8	$(CTC)_{5}$	5’-TGCAGGATTTATGTGATTGA-3’ 5’-CCCGGTGATGAATAGTCTAA-3’	50
315	PKSR T9	$(CAG)_{5}$	5’-CTTTGCTCTCCAGTTCAATC-3’ 5’-TAGATGATTTGCAGACGATG-3’	51
316	PKSR T10	$(CAA)_{6}$	5’-TCAGAAGAATTCGCAAAGAT-3’ 5’-TGACATAGCGAAGCTATGTG-3’	51
317	PKSR T11	$(CAG)_{6}$	5’-TCTAATGGTGTCGATTCCTC-3’ 5’-AAATGCTCAGTGTACAACCC-3’	52
318	PKSR T12	$(TCA)_{10}$	5’-AGCATTGCAGCCATTATTAT-3’ 5’-GGAAGAAATTTGTGAGCAAG-3’	50
319	PKSR T13	$(GGT)_{5}$	5’-TCATCATGACGTTCTTTTGA-3’ 5’-AACATGACATTGTCAAGCAA-3’	50

320	PKSR T14	$(TTC)_5$	5’-GACTGTTTGCAGTCTTCTCC-3’ 5’-ACAACCAACAAAAACTGGAC-3’	52
321	PKSR T15	$(TGG)_6$	5’-AAAGCAATGCTCTGTCTCAT-3’ 5’-TACAGTTCATCCGGGAATAC-3’	52
322	PKSS M1	$(T)_{12}$	5’-GGATTCCCTGAGTAAGAACC-3’ 5’-ATTTCCGGAATATGAGTGTG-3’	51
323	PKSS M2	$(A)_{14}$	5’-TAATCTAAAGGGAGTTCCCC-3’ 5’-AAACAATTTGAAAGAACCGA-3’	50
324	PKSS M3	$(T)_{12}$	5’-CCGGAAATACTAGGCCTACT-3’ 5’-CGATTGTCATAGGTATCTTTCA-3’	52
325	PKSS M4	$(T)_{14}$	5’-ACGACAAAAAGAACTCCTGA-3’ 5’-CTTTCAAGATTTATCGACCG-3’	50
326	PKSS M5	$(A)_{12}$	5’-AATAGGTGGGGTATTTCTCG-3’ 5’-CGTCTTTTTGAAGATCCAAG-3’	50
327	PKSS M6	$(A)_{12}$	5’-CCAACTATGTCAGGAAAAGG-3’ 5’-CGCGATTGATTTCTAAGATC-3’	50
328	PKSS M7	$(T)_{12}$	5’-GATTATGAACCAAAAACCGA-3’ 5’-ATTTATCCGCGATCTAGACA-3’	50
329	PKSS M8	$(A)_{12}$	5’-CAAATGTTCCGCTTTCTAAC-3’ 5’-AGTCGCTACGAGTCTACTGC-3’	53
330	PKSS M9	$(T)_{14}$	5’-GGCTCATTGGGATAGATGTA-3’ 5’-AAGAAAGTGGGGTAAAGACC-3’	52
331	PKSS M10	$(A)_{12}$	5’-TCAAGCAACAACAAAAAGAA-3’ 5’-ACCTTTTGTCGACCAAGATA-3’	50
332	PKSS M11	$(T)_{12}$	5’-TTCCTATCCCTGTCATGTTC-3’ 5’-GTTGAGGAAAGGGAGGATAT-3’	51
333	PKSS M12	$(T)_{12}$	5’-GTGCATCAGGAAAATTTAGC-3’ 5’-TGATCATGAGATCGTCAGAA-3’	50
334	PKSS M13	$(T)_{14}$	5’-TTCTGATTGATTGCCTTTTT-3’ 5’-TTCCTTTTCTTGCCATATGT-3’	49
335	PKSS M14	$(T)_{14}$	5’-CAATATCGAATCAATTCCGT-3’ 5’-GATGTCGTTATTCTTTTCGG-3’	49
336	PKSS M15	$(A)_{14}$	5’-TCAACTCCTGAAGATCAGCT-3’ 5’-GGTAGACTCGTGCACCATAT-3’	52
337	PKSS D1	$(AT)_7$	5’-GGCGTTACTCTACCACTGAG-3’ 5’-TTTTTCAATGAAATATCGTGAA-3’	49
338	PKSS D2	$(AT)_8$	5’-CTCTCAAGTCGAGACGACTC-3’ 5’-CCATTTTTGAAGTTACAGGC-3’	52
339	PKSS D3	$(TG)_7$	5’-GTACACCAAGAAAGAGGCTG-3’ 5’-TTCCGAGCATTACTGTTTCT-3’	50
340	PKSS D5	$(AG)_{10}$	5’-AAAGGAGTTTGGCTCTAGCT-3’ 5’-TACCCAGTACTACGGAAAGG-3’	51
341	PKSS D6	$(AT)_8$	5’-GTGAACGAGTTGATGGACTC-3’ 5’-GTTTTTCCCATGAAAACATG-3’	49
342	PKSS D7	$(TC)_7$	5’-GCTAAAGTGGGCTGTAGGTA-3’ 5’-AACTGCAAACAAACGGTAAT-3’	50
343	PKSS D8	$(AT)1_7$	5’-GATACAATTCAACCTTTCGG-3’ 5’-ACTTGGGCTTGTATTTGATG-3’	48
344	PKSS D9	$(AC)_{11}$	5’-TCTTCTCATTCCCATACACC-3’ 5’-GACTACGAACTTCTTGTGGC-3’	52
345	PKSS D10	$(AT)_{14}$	5’-TCTGAAGGTGTGTTGACTCA-3’ 5’-ACCTCAAAAACCAAATGAAA-3’	49
346	PKSS D11	$(AT)_{11}$	5’-TTTTTGTTACAAGCGGCTAT-3’ 5’-ACCAAATGAAACTGCAAATT-3’	53
347	PKSS D12	$(AG)_{16}$	5’-CAATTGACACTATGCACCAG-3’ 5’- GACTAAGCCTTCTAATGGCA -3’	52
348	PKSS D13	$(TA)_{10}$	5’-CCCTTTTGGATATTTTCACA-3’ 5’-ATCTGTAACCCTCTGCACTG-3’	53
349	PKSS D14	$(AG)_8$	5’-TCTATTTGATCGGTGGATTT-3’	52

			5’-CTCAGTAATTTGTGCCTTGC-3’	
350	PKSS T1	$(GGT)_5$	5’-GGACCTTCTGGGCTAAATAT-3’ 5’-AAATGGTGTTCCATTGGTAG-3’	51
351	PKSS T2	$(CAT)_5$	5’-CGTATCGAGAATGAAGGAAG-3’ 5’-AATGCTGTAAAGCACCAAAT-3’	50
352	PKSS T3	$(AAT)_{17}$	5’-GTTTGGTAGCATTTGAGAGG-3’ 5’-GTGGGCAGTTCATCCTAGTA-3’	52
353	PKSS T6	$(GAA)_5$	5’-CAGTGGATGTATTTGATCCC-3’ 5’-AACGTTTGAAGCAACAGAAT-3’	51
354	PKSS T7	$(TGC)_5$	5’-GCCTACAGCAGTATTTCCAG-3’ 5’-CACAACAACAGCAGGTTCTA-3’	53
355	PKSS T8	$(ACT)_{12}$	5’-AAACAAACCAACAAGTGGAG-3’ 5’-ATTGGATGGAAGGTTCTTCT-3’	51
356	PKSS T9	$(TCT)_5$	5’-TCCAACGAGCTTAATTTTGT-3’ 5’-ATCAACAGAGCGGAAAATTA-3’	50
357	PKSS T11	$(ATA)_{13}$	5’-CAACCCCAAAATAAGCTAGA 5’-AAGAGGAGGAGGATTTTCTG	51
358	PKSS T12	$(CAG)_5$	5’-CTCGAAGAATCCAAACTCAC-3’ 5’-GATTCTGACGTAATTGGAGC-3’	51
359	PKSS T13	$(ATA)_7$	5’-AATTAAAGAAACCGGGTTTC-3’ 5’-AAGAACAGACGCAATGATCT-3’	50
360	PKSS T14	$(ACC)_6$	5’-CCTCCTCCATTTCTCCTATC-3’ 5’-TATTCCCAATCCTTTGCTTA-3’	50

REFERENCES

[1] P. Bhandari, N. Kumar, B. Singh, A.P. Gupta, V.K. Kaul, and P.S. Ahuja, "Stability indicating LC-PDA method for determination of picrosides in hepatoprotective Indian herbal preparations of *Picrorhiza kurroa*", *Chromatographia*, vol. 69, pp. 221-227, 2008.

[2] V. Patial, K. Devi, M. Sharma, A. Bhattacharya, and P.S. Ahuja, "Propagation of *Picrorhiza kurroa* Royle ex Benth: An important medicinal plant of Western Himalaya", *Journal of Medicinal Plants Research*, vol. 6 (no. 34), pp. 4848-4860, 2012.

[3] B. Chandra, L.M.S. Palni, and S.K. Nandi, "Propagation and conservation of *Picrorhiza kurroa* Royle ex Benth: An endangered Himalayan medicinal herb of high commercial value", *Biodiversity and Conservation*, vol. 15, pp. 2325-2338, 2006.

[4] M.P. Nayar, and A.R.K. Sastri, "Red Data Plants of India," *CSIR Publication*, New Delhi, pp. 271, 1990.

[5] A. Shahzad, and T. Saeed, "In Vitro Conservation protocols for some rare medicinal plant species", *Recent Trends in Biotechnology and Therapeutic Applications of Medicinal Plants*, pp. 263-291, 2013.

[6] S. Dutt, G. Kiddle, B. Singh, B. Khambay, and C.H. Foyer, "Differential accumulation of picrosides in *Picrorhiza kurroa* Royle ex Benth plants", www.rothamstedinternational.org/files/posters/Posters/Sondutt/pdf, 2004.

[7] D. Rathee, M. Thanki, S. Bhuva, S. Anandjiwala, and R. Agrawal, "Iridoid glycosides-Kutkin, Picroside I and Kutkoside from *Picrorrhiza kurroa* Benth inhibits the invasion and migration of MCF-7 breast cancer cells through the down regulation of matrix metalloproteinases: 1st Cancer Update", *Arabian Journal of Chemistry*, vol. 6, pp. 49-58, 2013.

[8] R. Mehrotra, S. Rawat, D.K. Kulshreshtha, G.K. Patnaik, and B.N. Dhawan, "*In vitro* studies on the effect of certain natural products against hepatitis B virus", *Indian Journal of Medical Research*, vol. 92, pp. 133-138, 1990.

[9] Y. Zhou, "Use of kutkin I in preparing drug for treating hepatitis B", http://www.google.com/patents/CN100386087C, 2008.

[10] L. Wang, X. Liu, H. Chen, Z. Chen, X. Weng, T. Qiu, and L. Liu, "Effect of picroside II on apoptosis induced by renal ischemia/reperfusion injury in rats", *Experimental and Therapeutic Medicine,* vol. 9, pp. 817-822, 2015.

[11] L. Zhao, Y. Guo, X. Ji, and M. Zhang, "The neuroprotective effect of picroside II via regulating the expression of myelin basic protein after cerebral ischemia injury in rats", *BMC Neuroscience,* vol. 15, pp. 25, 2014.

[12] Y. Guo, X. Xu, Q. Li, Z. Li, and F. Du, "Anti-inflammation effects of picroside 2 in cerebral ischemic injury rats", *Behavioural and Brain Functions,* vol. 6, pp. 43, 2010.

[13] M. Zhang, P. Fangfang, R. Zhang, L. Zhao, and Y. Wu, "The antioxidant effect of picroside II and the optimizing of therapeutic dose and time window in cerebral ischemic injury in rats", *Merit Research Journal of Pharmacy and Pharmaceutical Sciences,* vol. 1, pp. 001-007, 2013.

[14] N. Wu, W. Li, W. Shu, and D. Jia, "Protective effect of picroside II on myocardial ischemia reperfusion injury in rats", *Drug Design Development and Therapy,* vol. 8, pp. 545–554, 2014.

[15] B.N. Dhawan, "Picroliv a new hepatoprotective agent from an Indian medicinal plant, *Picrorhiza kurroa*", *Medicinal Chemistry Research,* vol. 5, pp. 595-605, 1995.

[16] M. Katoch, I.S. Fazli, K.A. Suri, A. Ahuja, and G.N. Qazi, "Effect of altitude on picroside content in core collections of *Picrorhiza kurrooa* from the north western Himalayas", *Journal of Natural Medicine,* vol. 65, pp. 578-582, 2011.

[17] R. Kumar, P. Bhandari, B. Singh, and P.S. Ahuja, "Evaluation of *Picrorhiza kurrooa* accessions for growth and quality in north western Himalayas", *Journal of Medicinal Plants Research,* vol. 6 (no. 13), pp. 2660-2665, 2012.

[18] N. Sharma, V. Pathania, B. Singh, and R.C. Gupta, "Intraspecific variability of main phytochemical compounds in *Picrorhiza kurroa* Royle ex Benth from North Indian higher altitude Himalayas using reversed-phase high-performance liquid chromatography", *Journal of Medicinal Plants Research,* vol. 6 (no. 16), pp. 3181-3187, 2012.

[19] K. Weising, R.G. Atkinson, and R.C. Gardner, "Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation", *PCR Methods and Applications,* vol. 4, pp. 249-255, 1995.

[20] K. Weising, H. Nybom, K. Wolff, and W. Meyer, "DNA fingerprinting in Plants and Fungi", *Boca Raton,* FL: CRC Press, 1995.

[21] P. Poczai, I. Varga, N.E. Bell, and J. Hyvonen, "Genomics meets biodiversity: Advances in molecular marker development and their applications in plant genetic diversity assessment", in *The Molecular Basis of Plant Genetic Diversity*, Mahmut Caliskan (ed.), InTech, 2012, doi: 10.5772/33614.

[22] H. Sood, and R.S. Chauhan, "Biosynthesis and accumulation of a medicinal compound, Picroside-I, in cultures of *Picrorhiza kurroa* Royle ex Benth", *Plant Cell, Tissue and Organ Culture*, vol. 100, pp. 113-117, 2010.

[23] S. Pandit, K. Shitiz, H. Sood, and R.S. Chauhan, "Differential biosynthesis and accumulation of Picrosides in an endangered medicinal herb *Picrorhiza kurroa*", *Journal of Plant Biochemistry and Biotechnology*, vol. 22, pp. 335-342, 2012.

[24] H. Singh, P. Gahlan, and S. Kumar, "Cloning and expression analysis of ten genes associated with picrosides biosynthesis in *Picrorhiza kurroa*", *Gene*, pp. 320-328, 2012.

[25] S.S. Mahmoud, and R.B. Croteau, "Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase", *Proceedings of National Academy of Sciences of the United States of America*, vol. 98 (no. 15), pp. 8915-8920, 2001.

[26] D. Hampel, A. Mosandl, and M. Wust, "Biosynthesis of mono and sesquiterpenes in strawberry fruits and foliage: ^{2}H labelling studies", *Journal of Agriculture and Food Chemistry*, vol. 54, pp. 1473-1478, 2006.

[27] T. Kawoosa, H. Singh, A. Kumar, S.K. Sharma, K. Devi, S. Dutt, S.K. Vats, M. Sharma, P.S. Ahuja, and S. Kumar, "Light and temperature regulated terpene biosynthesis: hepatoprotective monoterpenepicroside accumulation in *Picrorhiza kurroa*", *Functional and Integrative Genomics*, vol. 10, pp. 393-404, 2010.

[28] V. Kumar, H. Sood, and R.S. Chauhan, "A proposed biosynthetic pathway of Picrosides linked through the detection of biochemical intermediates in the endangered medicinal herb *Picrorhiza kurroa*", *Phytochemical Analysis*, vol. 24, pp. 598-602, 2013.

[29] S.S. Mahmoud, and R.B. Croteau, "Strategies for transgenic manipulation of monoterpene biosynthesis in plants", *Trends in Plant Science*, vol. 7, pp. 366–373, 2002.

[30] C. Funk, and P.E. Brodelius, "Phenylpropanoid Metabolism in Suspension Cultures of *Vanilla planifolia* Andr: III. Conversion of 4-Methoxycinnamic Acids into 4-Hydroxybenzoic Acids", *Plant Physiology*, vol. 99, pp. 256-262, 1992.

[31] A. Narbad, and M.J. Gasson, "Metabolism of ferulic acid via vanillin using a novel CoA-dependent pathway in a newly isolated strain of *Pseudomonas fluorescens*", *Microbiology*, vol. 144, pp. 1397-1405, 1998.

[32] P. Gahlan, H.R. Singh, R. Shankar, N. Sharma, A. Kumari, V. Chawla, P.S. Ahuja, S. Kumar, "De novo sequencing and characterization of *Picrorhiza kurrooa* transcriptome at two temperatures showed major transcriptome adjustments", *BMC Genomics*, vol. 13, pp. 126, 2012.

[33] S. Pandit, K. Shitiz, P.K. Naik, H. Sood, and R.S. Chauhan, "Expression pattern of fifteen genes of non-mevalonate (MEP) and mevalonate (MVA) pathways in different tissues of endangered medicinal herb *Picrorhiza kurroa* with respect to Picrosides Content", *Molecular Biology Reports*, vol. 40, pp. 1053-63, 2013.

[34] W.W. Bhat, N. Dhar, S. Razdan, S. Rana, R. Mehra, A. Nargotra, R.S. Dhar, N. Ashraf, R. Vishwakarma, and S.K. Lattoo, "Molecular characterization of UGT94F2 and UGT86C4, two glycosyltransferases from *Picrorhiza kurrooa*: Comparative structural insight and evaluation of substrate recognition", *PLoS One*, vol. 8, pp. e73804, 2013.

[35] J. Palazon, R.M. Cusido, M. Bonfill, C. Morales, and M.T. Pinol, "Inhibition of paclitaxel and baccatin III accumulation bymevinolin and fosmidomycin in suspension cultures of *Taxus baccata*", *Journal of Biotechnology*, vol. 101, pp. 57-163, 2003.

[36] R.S. Singh, R.K. Gara, P.K. Bhardwaj, A. Kaachra, S. Malik, R. Kumar, M. Sharma, P.S. Ahuja, and S. Kumar, "Expression of 3-hydroxy-3-methylglutaryl-CoA reductase, p-hydroxybenzoate-m-geranyltransferase and genes of phenylpropanoid pathway exhibits positive correlation with shikonins content in arnebia [*Arnebia euchroma* (Royle) Johnston]", *BMC Molecular Biology*, vol. 11, pp. 88, 2010.

[37] R. Croteau, T.M. Kutchan, and N.G. Lewis, "Natural products (Secondary metabolites)", in *Biochemistry and Molecular Biology of Plants*, B. Buchanan, W. Gruissem, and R. Jones (eds.), American Society of Plant Physiologists (Rockville, Madison), 2000, pp. 1250-1318.

[38] J.J.B. Keurentjes, "Genetical metabolomics: closing in on phenotypes", *Current Opinion in Plant Biology*, vol. 12, pp. 223-230, 2009.

[39] F.M. El-Domyati, R.A.A. Younis, S. Edris, A. Mansour, J. Sabir, and A. Bahieldi, "Molecular markers associated with genetic diversity of some medicinal plants in Sinai", *Journal of Medicinal Plants Research*, vol. 5 (no. 10), pp. 1918-1929, 2011.

[40] J.J. Gutierrez-Gonzalez, X. Wu, J. Zhang, et al., "Genetic control of soybean seed isoflavone content: importance of statistical model and epistasis in complex traits", *Theoretical and Applied Genetics*, vol. 119, pp. 1069-1083, 2009.

[41] K. Saito, and F. Matsuda, "Metabolomics for functional genomics, systems biology, and biotechnology", *Annual Review of Plant Biology*, vol. 61, pp. 463-489, 2010.

[42] J.F. Royle, "Illustrations of the botany and other branches of the natural history of the Himalayan mountains, and of the flora of Cashmere", Volume plates, Reprint 1970. Today and Tomorrow's Printers and Publishers, New Delhi, p 71, 1935.

[43] W.W. Smith, and G.H. Cave, "The vegetation of the Zemu and Llonakh Valleys of Sikkim", *Recordings* of *The Botanical Survey of India*, vol. 4, pp. 141–260, 1911.

[44] F.W. Pennell, "The Scrophulariaceae of the Western Himalayas", Bishen Singh Mahendra Pal Singh, Dehra Dun, pp 63-66 (reprint 1997), 1943.

[45] P.K. Pusalkar, "*Picrorhiza tungnathii* sp. nov. from western Himalaya, India", *Nordic Journal of Botany*, vol. 32, pp. 308-313, 2014.

[46] R. Raina, T.S. Mehra, R. Chand, and Y.P. Sharma, "Reproductive Biology of *Picrorhiza kurroa* - a Critically Endangered High Value Temperate Medicinal Plant" *Open Access Journal of Medicinal and Aromatic Plants*, vol. 1 (no. 2), pp. 40-43, 2010.

[47] A. Nag, S. Chanda, and S. Rajkumar, "Estimation of nuclear genome size of important medicinal plant species from western Himalaya using flow cytometry", *Journal of Cell and Plant Sciences*, vol. 2 (no. 2), pp. 19-23, 2011.

[48] K. Weinges, P. Kloss, and W.D. Henkels, "Picrosid-II, ein neues 6-vanilloyl-catalpol aus *Picrorhiza kurroa* Royle und Benth", *Liebigs Annalen der Chemie*, vol. 759, pp. 173-182, 1972.

[49] H. Stuppner, and H. Wagner, "New cucurbitacin glycosides from *Picrorhiza kurroa*", *Planta Medica*, vol. 55, pp. 559-563, 1989.

[50] I. Kitagawa, K. Hino, T. Nishimura, E. Iwata, and I. Yosioka, "On the constituents of *Picrorhiza kurroa*. (1) The structure of picroside I, a bitter principle of the subterranean part", *Chemical and Pharmaceutical Bulletein*, vol. 19, pp. 2534-2544, 1971.

[51] K. Weinges, and K. Künstler, "Notiz über die Isolierung und Konstitutionsaufklärung eines neuen Picrosids aus *Picrorhiza kurroa* Royle und Benth", *Liebigs Annalen der Chemie*, pp. 1053-1057, 1977.

[52] J.X. Li, P. Li, Y. Tezuka, T. Namba, and S. Kadota, "Three phenylethanoid glycosides and an iridoid glycoside from *Picrorhiza scrophulariiflora*", *Phytochemistry,* vol. 48, pp. 537-542, 1998.

[53] J.M. Simons, "Immunomodulation by *Picrorhiza kurroa* - basis for its ethnomedical use?", Ph.D. thesis, Rijksuniversiteit Utrecht, Utrecht, 1989.

[54] D.Q. Wang, Z.D. He, B.S. Feng, and C.R. Yang, "Chemical constituents from *Picrorhiza scrophulariiflora*", *Acta Botanica Yunnanica,* vol. 15, pp. 83-88, 1993.

[55] Q. Jia, M.F. Hong, and D. Minter, "Pikuroside: a novel iridoid from *Picrorhiza kurroa*", *Journal of Natural Products,* vol. 62, pp. 901-903, 1999.

[56] B. Singh, and R.P. Rastogi, "Chemical examination of *Picrorhiza kurroa* Benth. Part VI. Reinvestigation of kutkin", *Indian Journal of Chemistry,* vol. 10, pp. 29-31, 1972.

[57] H. Stuppner, H. Kählig, O. Seligmann, and H. Wagner, "Minor cucurbitacin glycosides from *Picrorhiza kurrooa*", *Phytochemistry,* vol. 29, pp. 1633-1637, 1990.

[58] R.P Rastogi, V.N. Sharma, and S. Siddiqui, "Chemical examination of *Picrorhiza kurroa* Benth - Part 1", *Journal of Scientific and Industrial Research,* vol. 8B, pp. 173-178, 1949.

[59] K. Basu, B. Dasgupta, S.K. Bhattacharya, and P.K. Debnath, "Chemistry and pharmacology of apocynin, isolated from *Picrorhiza kurrooa* Royle ex Benth". *Current Science,* vol. 22, pp. 603-604, 1971.

[60] J.C. Sacchettini, and C.D. Poulter, "Creating isoprenoid diversity", *Science,* vol. 277, pp. 1788-1789, 1997.

[61] R.A. Ansari, B.S.Aswal, R. Chander, B.N. Dhawan, N.K. Garg, N.K. Kapoor, D.K. Kulshreshta, H. Mehdi, B.N.Mehrotra, G.K. Patnaik, and S.K. Sharma, "Hepatoprotective activity of kutkin, the iridoid glycoside mixture of *Picrorhiza kurroa*", *Indian Journal of Medical Research,* vol. 87, 401-404, 1988.

[62] A.K. Dwivedi, M. Chaudry, R.K. Seth, and J.P.S. Sarin, "Simultaneous micro-determination of picroside I and kutkoside in Picroliv (kutkin) by high performance liquid chromatography", *Indian Journal of Pharmaceutical Sciences,* pp. 274-275, 1989.

[63] H.F. Smit, "*Picrorhiza scrophulariiflora,* from traditional use to immunomodulatory activity," University of Utrecht, Faculty of Pharmacy, Utrecht, 2000.

[64] R.W. Fuller, J.H. Cardellina II, G.M. Cragg, and M.R. Boyd, "Cucurbitacins: differential cytotoxicity, dereplication and first isolation from *Gonystylus keithii*", *Journal of Natural Products*, vol. 57, 1442-1445, 1994.

[65] E. Steinegger, and R. Hänsel, "*Lehrbuch der Pharmakognosie und Phytopharmazie*", Fourth edition, Springer Verlag, Berlin, 1988.

[66] B. Saraswat, P.K.S. Visen, G.K. Patnaik, and B.N. Dhawan, "Ex vivo and in vivo investigations of picroliv from *Picrorhiza kurroa* in an alcohol intoxication model in rats", *Journal of Ethnopharmacology*, vol. 66, pp. 263-269, 1999.

[67] A.B. Vaidya, D.S. Antarkar, J.C. Doshi, A.D. Bhatt, V.V. Ramesh, P.V. Vora, D.D. Perissond, A.J. Baxi, and P.M. Kale, "*Picrorhiza kurroa* (Kutaki) Royle ex Benth as a hepatoprotective agent-experimental & clinical studies", *Journal of Postgraduate Medicine*, vol. 42, pp. 105, 1996.

[68] S.N. Shetty, S. Mengi, R. Vaidya, and A.D.B. Vaidya, "A study of standardized extracts of *Picrorhiza kurroa* Royle ex Benth in experimental nonalcoholic fatty liver disease", *Journal of Ayurveda and Integrative Medicine*, vol. 1 (no. 3), pp. 203-210, 2010.

[69] V. Rajkumar, G. Guha, and R.A. Kumar, "Antioxidant and anti-neoplastic activities of *Picrorhiza kurroa* extracts", *Food and Chemical Toxicology*, vol. 49, pp. 363-369, 2011.

[70] K. Kant, M. Walia, V.K. Agnihotri, V. Pathania, and B. Singh, "Evaluation of Antioxidant Activity of *Picrorhiza kurroa* (Leaves) Extracts", *Indian Journal of Pharmaceutical Sciences*, vol. 75 (no. 3), pp. 324-329, 2013.

[71] A. Ray, S.R. Chaudhuri, B. Majumdar, and S.K. Bandyopadhyay, "Antioxidant activity of ethanol extract of rhizome of *Picrorhiza kurroa* on indomethacin induced gastric ulcer during healing", *Indian Journal of Clinical Biochemistry*, vol. 17 (no. 2), pp. 44-51, 2002.

[72] A. Gupta, A. Khajuria, J. Singh, K.L. Bedi, N.K. Satti, P. Dutt, K.A. Suri, O.P. Suri, and G.N. Qazi, "Immunomodulatory activity of biopolymeric fraction RLJ-NE-205 from *Picrorhiza kurroa*", *International Immunopharmacology*, vol. 6, pp. 1543-1549, 2006.

[73] A. Gupta, A. Khajuria, J. Singh, and K.A. Suri, "Adjuvant effect of biopolymeric fraction from *Picrorhiza kurroa* to promote both Th1 and Th2 immune responses", *Journal of Progressive Research in Biology*, vol. 1 (no. 1), pp. 7-16, 2015.

[74] K.L. Joy, N.V. Rajeshkumar, G. Kuttan, and R. Kuttan, "Effect of *Picrorrhiza kurroa* extract on transplanted tumours and chemical carcinogenesis in mice", *Journal of Ethnopharmacology* , vol. 71, pp. 261-266, 2000.

[75] V. Singh, and H.S. Banyal, "Antimalarial effects of *Picrorhiza kurrooa* Royle ex Benth extracts on *Plasmodium berghei*", *Asian Journal of Experimental Biologocal Sciences,* vol. 2 (no. 3), pp. 529-532, 2011.

[76] N. Kumar, and S.K. Sharma, "Antimalarial potential of *Picrorrhiza kurroa* Royle ex Benth rhizome using mice", *Asian Pacific Journal of Tropical Biomedicine,* vol. 4 (no.2), pp. S769-S773, 2014.

[77] S.K. Sharma, and N. Kumar, "Antimicrobial screening of *Picrorhiza kurroa* Royle ex Benth rhizome, *International Journal of Current Pharmaceutical Review and Research*, vol. 3 (no. 3), pp. 60-65, 2012.

[78] D.K. Zhang, J.J. Yu, Y.M. Li, L.N. Wei, Y. Yu, Y.H. Feng, and X. Wang, "A *Picrorhiza kurroa* derivative, Picroliv, attenuates the development of dextran-sulfate-sodium-induced colitis in mice", *Mediators of Inflammation,* vol. 2012, pp. 1-9, 2012.

[79] M. Singh, V. Tiwari, A. Jain, and S. Ghoshal, "Protective activity of picroliv on hepatic amoebiasis associated with carbon tetrachloride toxicity", *Indian Journal of Medical Research*, vol. 121, pp. 678-682, 2005.

[80] N. Mittal, N. Gupta, S. Saksena, N. Goyal, U. Roy, and A. K. Rastogi, "Protective effect of picroliv from *Picrorhiza kurroa* against *Leshmania donovani* infections in *mesocricetus auratus*", *Life Sciences*, vol. 63 (no. 22), pp. 1823-1834, 1998.

[81] S. Sinha, J. Mehrotra, L. Bala, A.K. Jaiswal, and B.N. Dhawan, "Picroliv, the iridoid glycoside fraction of *Picrorhiza kurroa*, selectively augments human T cell response to mycobacterial protein antigens", *Immunopharmacology and Immunotoxicology,* vol. 20 (no. 4), pp. 579-588, 1998.

[82] S.A. Sane, N. Shakya, and S. Gupta, "Immunomodulatory effect of picroliv on the efficacy of paromomycin and miltefosine in combination in experimental visceral leishmaniasis", *Experimental Parasitology,* vol. 127, pp. 376–381, 2011.

[83] G.M. Husain, P.N. Singh, and V. Kumar, "Antidiabetic activity of standardized extract of *Picrorhiza kurroa* in rat model of NIDDM", *Drug Discovery and Therapeutics,* vo. 3 (no. 3), pp. 88-92, 2009.

[84] S. Sinha, J. Bhat, M. Joshi, V. Sinkar, and S. Ghaskabdi, " Hepatoprotective activity of *Picrorhiza kurroa* Royle Ex. Benth extract against alcohol cytotoxicity in mouse

liver slice culture", *International Journal of Green Pharmacy*, vol. 5 (no. 3), pp. 244-253, 2011.

[85] W. Dorch, H. Stuppner, H. Wagner, M. Gropp, S. Demoulin, and J. Ring, "Antiasthmatic effects of *Picrorhiza kurroa* : Androsin prevents allergen- and PAF-induced bronchial bbstruction in guinea pigs", *International Archives of Allergy and Applied Immunology*, vol. 95, pp. 128-133, 1991.

[86] S. Irshad, A. Mannan, and B. Mirza, "Antimalarial activity of three pakistani medicinal plants", *Pakistan Journal of Pharmaceutical Sciences*, vol. 24 (no. 4), pp. 589-591, 2011.

[87] D.K. Zhang, J.J. Yu, Y.M. Li, L.N. Wei, Y. Yu, Y.H. Feng and, X. Wang, "A *Picrorhiza kurroa* derivative, picroliv, attenuates the development of dextran-sulfate-sodium-induced colitis in mice," *Mediators of Inflammation*, vol. 2012, pp. 751629, 2012.

[88] P. Li, K. Matsunaga, T. Yamakuni, and Y. Ohizumi, "Potentiation of nerve growth factor-action by picrosides I and II, natural iridoids, in PC12D cells", *European Journal of Pharmacology*, vol. 406 (no. 2), pp. 203-208, 2000.

[89] P. C. Verma, V. Basu, V. Gupta, G. Saxena, and L. ur Rahman, "Pharmacology and Chemistry of a Potent Hepatoprotective Compound Picroliv Isolated from the Roots and Rhizomes of *Picrorhiza kurroa* Royle ex Benth. (Kutki)", *Current Pharmaceutical Biotechnology*, vol. 10 (no. 6), pp. 641-649, 2009.

[90] G. Yang, R. Zhang, X. Li, and Q. Sui, "Picroside II could protect the cerebral ischemic injury by reducing the content of free radical and enhancing the activity of antioxidase in rats", *Journal of Advances in Biology*, vol. 3 (no. 2), pp. 219-226, 2014.

[91] Y. Wang, R. Zhang, L. Zhong, G. Liu, and X.N. Ji, "Picroside II could reduce the concentration of NO and enhance the activity of GSHPx in cerebral ischemic injury in rats", *Research in Neurology: An International Journal*, vol. 2014, pp. 1-8, 2014.

[92] H. Gao, and Y.W. Zhou, "Anti-lipid peroxidation and protection of liver mitochondria against injuries by picroside II", *World Journal of Gastroenterology*, vol. 11 (no. 24), pp. 3671-3674, 2005.

[93] H. Gao, and Y.W. Zhou, "Inhibitory effect of picroside II on hepatocyte apoptosis", *Acta Pharmacologica Sinica*, vol. 26 (no. 6), pp. 729–736, 2005.

[94] Q. Li, Z. Li, X.Y. Xu, Y.L. Guo, and F. Du, "Neuroprotective Properties of Picroside II in a Rat Model of Focal Cerebral Ischemia", *International Journal of Molecular Sciences*, vol. 11 (no. 11), pp. 4580-4590, 2010.

[95] F.J. Meng, S.M. Jiao, and B. Yu, "Picroside II protects cardiomyocytes from hypoxia/reoxygenation induced apoptosis by activating the PI3K/Akt and CREB pathways", *International Journal of Molecular Medicine*, vol. 30 (no. 2), pp. 263-270, 2012.

[96] F. Engels, B.F. Renirie, B.A. t' Hart, R.P. Labadie, and F.P. Nijkamp, "Effects of apocynin, a drug isolated from the roots of *Picrorhiza kurroa*, on arachidonic acid metabolism", *FEBS Journal*, vol. 305 (no. 3), pp. 254-256, 1992.

[97] R.M. Touyz, "Apocynin, NADPH oxidase, and vascular cells-a complex matter", *Hypertension*, vol. 51, 172-174, 2008.

[98] J. Zhu, X. Chen, H. Wang, and Q. Yan, "Catalpol protects mice against renal ischemia/reperfusion injury via suppressing PI3K/Akt-eNOS signaling and inflammation", *International Journal of Clinical and Experimental Medicine*, vol. 8 (no. 2), pp. 2038-2044, 2015.

[99] Y.R. Liu, R.Y. Lei, C.E. Wang, B.A. Zhang, H. Lu, H.C. Zhu, and G.B. Zhang, "Effects of catalpol on ATPase and amino acids in gerbils with cerebral ischemia/reperfusion injury", *Neurological Sciences*, vol. 35, pp. 1229-1233, 2014.

[100] J.H. Wang, H. Xie, T.K. Zhao, B. Kang, "Catalpol regulates cholinergic nerve system function through effect on choline acetyl-transferase not M receptor affinity", *Biomedicine and Pharmacotherapy*, http://dx.doi.org/10.1016/j.biopha.2014.12.014, 2014.

[101] H. Pei, X. Su, L. Zhao, H. Li, Y. Guo, M. Zhang, and H. Xin, "Primary study for the therapeutic dose and time window of picroside II in treating cerebral ischemic injury in rats", *International Journal of Molecular Sciences*, vol. 13, pp. 2551-2562, 2012.

[102] R. Bauer, "Quality criteria and standardization of phytopharmaceuticals: Can acceptable drug standards be achieved," *Drug Information Journal*, vol. 32, pp. 101-110, 1998.

[103] D.K. Ved, and G.S. Goraya, "Demand and Supply of Medicinal Plants in India", Foundation for Revitalisation of Local Health Traditions (Bangalore, India), National Medicinal Plants Board, Bishen Singh, Mahendra Pal Singh, 2008.

[104] A. Uniyal, and S.K Uniyal, "Distribution, status and conservation of *Picrorhiza kurrooa* in the Himalayan region" in: G.S. Rawat, Editor, *Special Habitats and Threatened Plants of India*, Wildlife Institute of India, Dehradun, India, 2008.

[105] M. Katoch, I.S. Fazli, K.A. Suri, A. Ahuja, and G.N. Qazi, "Effect of altitude on picroside content in core collections of *Picrorhiza kurrooa* from the north western Himalayas", *Journal of Natural Medicine*, vol. 65, pp. 578-582, 2011.

[106] H. Lohani, G. Gwari, H.C. Andola, and N. Chauhan, "High performance liquid chromatographic determination of Picroside I & II in *Picrorhiza kurroa* Royle ex Benth: A critically endangered plant of Himalaya", *Medicinal Plants*, vol. 3 (no. 2), pp. 165-167, 2011.

[107] L. Olofsson, A. Engström, A. Lundgren, P.E. Brodelius, "Relative expression of genes of terpene metabolism in different tissues of *Artemisia annua* L", *BMC Plant Biology*, vol. 11, pp. 45, 2011.

[108] S.E. O'Connor, "Elucidation of natural product pathways in plants", in *Plant-Derived Natural Products; Synthesis, function and application*, A.E. Osbourn, and V. Lanzotti (eds.), Springer Dordrecht Heidelberg London New York, 2009, doi: 10.1007/978-0-387-85498-4

[109] M.Y. Hirai, M. Klein, Y. Fujikawa, et al., "Elucidation of gene-to-gene and metabolite-to-gene networks in *Arabidopsis* by integration of metabolomics and transcriptomics", *The Journal of Biological Chemistry*, vol. 280 (no. 27), pp. 25590-25595, 2005.

[110] O. Morozova, M. Hirst, and M.A Marra, "Applications of new sequencing technologies for transcriptome analysis", *Annual Review of Genomics and Human Genetics*, vol. 10, pp. 135-151, 2009.

[111] J. Shendure, and H. Ji, "Next-generation DNA sequencing", *Nature Biotechnology*, vol. 26, pp. 1135-1145, 2008.

[112] A.L. Eveland, D.R. McCarty, and K.E. Koch, "Transcript profiling by 3 - untranslated region sequencing resolves expression of gene families", *Plant Physiology*, vol. 146, pp. 32-44, 2008.

[113] R. Li, Y. Li, X. Fang, H. Yang, J. Wang, and K. Kristiansen, "SNP detection for massively parallel whole-genome resequencing", *Genome Research*, vol. 19, pp. 1124-1132, 2009.

[114] A. Sud, R.S. Chauhan, and C. Tandon, "Identification of imperative enzymes by differential protein expression in *Picrorhiza kurroa* under metabolite accumulating

and non-accumulating conditions", *Protein & Peptide Letters*, vol. 20, pp. 826-835, 2013.

[115] A. Sud, R.S. Chauhan, and C. Tandon, "Mass spectrometric analysis of differentially expressed proteins in an endangered medicinal herb, *Picrorhiza kurroa*", *BioMed Research International*, vol. 2014, http://dx.doi.org/10.1155/2014/326405, 2014.

[116] W.W. Bhat, S. Razdan, S. Rana, N. Dhar, T.A. Wani, P. Qazi, R. Vishwakarma, and S.K. Lattoo, "A phenylalanine ammonia-lyase ortholog (PkPAL1) from *Picrorhiza kurrooa* Royle ex. Benth: molecular cloning, promoter analysis and response to biotic and abiotic elicitors", *Gene*, http://dx.doi.org/10.1016/j.gene.2014.06.046, 2014.

[117] V. Kumar, K. Shitiz, R.S. Chauhan, H. Sood, and C. Tandon, "Tracking dynamics of enzyme activities and their gene expression in *Picrorhiza kurroa* with respect to picroside accumulation", *Journal of Plant Biochemistry and Biotechnology*, 10.1007/s13562-015-0317-7, 2015.

[118] M.K. Dhar, B. Friebe, S. Kaul, and B.S. Gill, "Characterization and physical mapping of 18S - 5.8S - 25S and 5S ribosomal RNA gene families in *Plantago* species", *Annals of Botany*, vol. 97, pp. 541–548, 2006.

[119] S.P.S. Khanuja, S. Paul, A.K. Shasany, A.K. Gupta, M.P. Darokar, et al., "Genetically tagged improved variety 'CIM-Arogya' of *Artemisia annua* for high artemisinin yield", *Journal of Medicinal and Aromatic Plant Sciences*, vol. 27, pp. 520-524, 2005.

[120] M. Morgante, M. Hanafey, and W. Powell, "Microsatellites are preferentially associated with nonrepetitive DNA in plant genomes" *Nature Genetics*, vol. 30, pp. 194-200, 2002.

[121] Y. Zhu, J.E, Strassmann, and D. Queller, "Insertions substitutions and origin of microsatellites", *Genome Research*, vol. 76, pp. 227-236, 2000.

[122] J.M. Hancock, and M.F. Santibanez-Koref, "Trinucleotide expansion diseases in the context of micro- and minisatellite evolution, *The EMBO Journal*, vol. 17, pp. 5521-5524, 1998.

[123] Y.J. Park, J.K. Lee, and N.S. Kim, "Simple sequence repeat polymorphisms (SSRPs) for evaluation of molecular diversity and germplasm classification of minor crops", *Molecules*, vol. 14, pp. 4546-4569, 2009.

[124] D.J. Kliebenstein, J. Kroymann, and P. Brown, et al., "Genetic control of natural variation in *Arabidopsis thaliana* glucosinolate accumulation", *Plant Physiology*, vol. 126, pp. 811-825, 2001.

[125] C.V. Acuña, P.V. Villalba, M. García, P. Pathauer, H.E. Hopp, and S.N.M. Poltri, "Microsatellite markers in candidate genes for wood properties and its application in functional diversity assessment in *Eucalyptus globulus*", *Electronic Journal of Biotechnology*, vol. 15 (no. 2), 2012.

[126] T. Sotelo, P. Soengas, P. Velasco, V.M. Rodríguez, and M.E. Cartea, "Identification of metabolic qtls and candidate genes for glucosinolate synthesis in *Brassica oleracea* leaves, seeds flower buds", *PLoS One*, vol. 9 (no. 3), pp. e91428, 2014.

[127] D.P. del Carpio, R.K. Basnet, D. Arends, et al., "Regulatory Network of Secondary Metabolism in *Brassica rapa*: Insight into the Glucosinolate Pathway", *PLoS One*, vol. 9 (no. 9), pp. e107123, 2014.

[128] M.A. Hussain, R.K. Aggarwal, Y.S. Bedi, and M.Z. Abdin, "Microsatellite DNA marker isolation from *Picrorhiza kurrooa* Royle ex Benth by magnetic capture", *Journal of Applied Biosciences*, vol. 21, pp. 1237-1245, 2009.

[129] M. Katoch, M.A. Hussain, and A. Ahuja, "Comparison of SSR and cytochrome P-450 markers for estimating genetic diversity in *Picrorhiza kurrooa* L.", *Plant Systematics and Evolution*, vol. 299, pp. 1637–1643, 2013.

[130] T. Murashige, and F. Skoog, "A revised medium for rapid growth and bioassay with tobacco tissue cultures", *Physiologia Plantarum*, vol. 15, pp. 473-497, 1962.

[131] B. Li, and C.N. Dewey, "RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome", *BMC Bioinformatics*, vol. 12, pp. 323, 2011.

[132] M.G. Murray, and W.F. Thompson, "Rapid isolation of high molecular weight plant DNA", *Nucleic Acids Research*, vol. 8 (no. 19), pp. 4321-4325, 1980.

[133] J.K. Pritchard, M. Stevens, and P. Donnelly, "Inference of population structure using multilocus genotype data", *Genetics*, vol. 155, pp. 945-959, 2000.

[134] X. Perrier, and J.P. Jacquemoud-Collet, "DARwin software" http://darwin.cirad.fr/darwin, 2006.

[135] F.C. Yeh, R.C. Yang, and T. Boyle, "POPGENE 32-version 1.31, Population Genetics Software", http.//www.ualberta.ca/~fyeh/fyeh/, 1999.

[136] T.C. Marshall, J. Slate, L.E.B. Kruuk, and J.M. Pemberton, "Statistical confidence for likelihood-based paternity inference in natural populations". *Molecular Ecology*, vol. 7, pp. 639-655, 1998.

[137] K. Miettinen, L. Dong, N. Navrot, et al., "The seco-iridoid pathway from *Catharanthus roseus*", *Nature Communications*, vol. 5, pp. 3606, 2014.

[138] M. Nagatoshi, K. Terasaka, A. Nagatsu, and H. Mizukami, "Iridoid-specific glucosyltransferase from *Gardenia jasminoides*", *The Journal of Biological Chemistry*, vol. 286 (no. 37), pp. 32866-32874, 2011.

[139] K. Shinozaki, and E.S. Dennis, "Cell signalling and gene regulation: global analyses of signal transduction and gene expression profiles", *Current Opinion in Plant Biology*, vol. 6, pp. 405-409, 2003.

[140] C. Bodeker, G. Bodeker, C.K. Ong, C.K. Grundy, G. Burford, and K. Shein, "WHO Global atlas of traditional, complementary and alternative medicine", World Health Organization, Geneva, 2005.

[141] O.F. Kunle, H.O. Egharevba, and P.O. Ahmadu, "Standardization of herbal medicines - A review", *International Journal of Biodiversity and Conservation*, vol. 4 (no. 3), pp. 101-112, 2012.

[142] A. Uniyal, S.K. Uniyal, and G.S. Rawat, "Commercial Extraction of *Picrorhiza kurrooa* Royle ex Benth. in the Western Himalaya", *Mountain Research and Development*, vol. 31 (no. 3), pp. 201-208, 2011.

[143] R. Kowalski, and T. Wolski, "Evaluation of phenolic acid content in *Silphium perfoliatum* L., leaves, inflorescences and rhizomes," *Electronic Journal of Polish Agricultural Universities*, vol. 6 (no. 1), 2006.

[144] N. Praveen, P.M. Naik, S.H. Manohar, and H.N. Murthy, "Distribution of withanolide A content in various organs of *Withania somnifera* (L.) Dunal", *International Journal of Pharmacy and Biological Sciences*, vol. 1, pp. 1-5, 2010.

[145] N. Malhotra, V. Kumar, H. Sood, T.R. Singh, and R.S. Chauhan, "Multiple genes of mevalonate and non-mevalonate pathways contribute to high aconites content in an endangered medicinal herb, *Aconitum heterophyllum* Wall", *Phytochemistry*, vol. 108, pp. 26-34, 2014.

[146] J.K. Padahan, V. Kumar, H. Sood, T.R. Singh, and R.S. Chauhan, "Contents of therapeutic metabolites in *Swertia chirayita* correlate with the expression profiles of multiple genes in corresponding biosynthesis pathways", *Phytochemistry*, http://dx.doi.org/10.1016/j.phytochem.2015.05.007, 2015.

[147] S. Zhao, C.H. Park, X. Li, et al., "Accumulation of rutin and betulinic acid and expression of phenylpropanoid and triterpenoid biosynthetic genes in mulberry (*Morus alba* L.)", *Journal of Agricultural and Food Chemistry*, doi: 10.1021/acs.jafc.5b03221, 2015.

[148] D. Kliebenstein, "Quantitative genomics: analyzing intraspecific variation using global gene expression polymorphisms or eQTLs", *Annual Review of Plant Biology*, vol. 60, pp. 93–114, 2009.

[149] P. Kumar, T. Pal, N. Sharma, V. Kumar, H. Sood, and R.S. Chauhan, "Expression analysis of biosynthetic pathway genes vis-à-vis podophyllotoxin content in *Podophyllum hexandrum* Royle", *Protoplasma*, 10.1007/s00709-015-0757-x, 2015.

[150] A.J. Simkin, K. Miettinen, P. Claudel, et al., "Characterization of the plastidial geraniol synthase from Madagascar periwinkle which initiates the monoterpenoid branch of the alkaloid pathway in internal phloem associated parenchyma", *Phytochemistry*, vol. 85, pp. 36-43, 2013.

[151] G. Collu, N, Unver, A.M.G. Peltenburg-Looman, R. van der Heijden, R. Verpoorte, and J. Memelink, "Geraniol 10-hydroxylase, a cytochrome P450 enzyme involved in terpenoid indole alkaloid biosynthesis", *FEBS Letters*, vol. 508, pp. 215-220, 2001.

[152] J. Wang, Y. Liu, Y. Cai, F. Zhang, G. Xia, and F. Xiang, "Cloning and functional analysis of geraniol 10-hydroxylase, a cytochrome P450 from *Swertia mussotii* Franch", *Bioscience, Biotechnology and Biochemistry*, vol. 74, (no. 8), pp. 1583-1590, 2010.

[153] R. Krithika, P.L. Srivastava, B. Rani, S.P. Kolet, M. Chapade, M. Soniya, and H.V. Thulasiram, "Characterization of 10-hydroxygeraniol dehydrogenase from *Catharanthus roseus* reveals cascaded enzymatic activity in iridoid biosynthesis", *Scientific Reports*, vol. 5, pp. 8258, 2015.

[154] F. Geu-Flores, N.H. Sherden, V. Courdavault, et al., "An alternative route to cyclic terpenes by reductive cyclization in iridoid biosynthesis", *Nature*, vol. 492, pp. 138-142, 2012.

[155] X. Li, Y. Li, D. Wei, P. Li, L. Wang, and L. Feng, "Characterization of a broad-range aldehyde dehydrogenase involved in alkane degradation in *Geobacillus thermodenitrificans* NG80-2", *Microbiological Research*, vol. 165, pp. 706-712, 2010.

[156] M. Ranjbar, M.R. Naghavi, H. Alizadeh, and H. Soltanloo, “Expression of artemisinin biosynthesis genes in eight *Artemisia* species at three developmental stages”, *Industrial Crops and Products*, vol. 76, pp. 836-843, 2015.

[157] R. Koncitikova, A. Vigouroux, M. Kopecna, et al., “Role and structural characterization of plant aldehyde dehydrogenases from family 2 and family 7”, *Biochemical Journal*, vol. 468, pp. 109-123, 2015.

[158] N. Stiti, T.D. Missihoun, S.O. Kotchoni, H.H. Kirch, and D. Bartels, “Aldehyde dehydrogenases in *Arabidopsis thaliana*: biochemical requirements, metabolic pathways, and functional analysis”, *Frontiers in Plant Science*, vol. 2, pp. 65, 2011.

[159] F. Wellman, U. Matern, and R. Lukacin, “Significance of C-terminal sequence elements for *Petunia* flavanone 3β-hydroxylase activity”, *FEBS Letters*, vol. 561, 149-154, 2004.

[160] G. Shen, Y. Pang, W. Wu, et al., “Cloning and characterization of a flavanone 3-hydroxylase gene from *Ginkgo biloba*”, *Bioscience Reports*, vol. 26, pp. 19-29, 2006.

[161] K. Singh, A. Rani, S. Kumar, et al., “An early gene of the flavonoid pathway, flavanone 3hydroxylase, exhibits a positive relationship with the concentration of catechins in tea (*Camellia sinensis*)”, *Tree Physiology*, vol. 28, pp. 134956, 2008.

[162] I. Beritognolo, E. Magel, A. Abdel-Latif, J.P. Charpentier, C. Jay-Allemand, and C. Breton, “Expression of genes encoding chalcone synthase, flavanone 3-hydroxylase and dihydroflavonol 4-reductase correlates with flavanol accumulation during heartwood formation in *Juglans nigra*”, *Tree Physiology*, vol. 22 (no. 5), pp. 291-300, 2002.

[163] T. Hakamatsuka, K. Mori, S. Ishida, Y. Ebizuka, and U. Sankawa, “Purification of 2-hydroxyisoflavanone dehydratase from the cell cultures of *Pueraria lobata*”, *Phytochemistry*, vol. 49 (no. 2) pp. 497-505, 1998.

[164] T. Akashi, T. Aoki, and S. Ayabe, “Molecular and biochemical characterization of 2-hydroxyisoflavanone dehydratase. Involvement of carboxylesterase-like proteins in leguminous isoflavone biosynthesis”, *Plant Physiology*, vol. 137, pp. 882-891, 2005.

[165] M. Shimamura, T. Akashi, N. Sakurai, H. Suzuki, K. Saito, D. Shibata, S. Ayabe, and T. Aoki, “2-Hydroxyisoflavanone dehydratase is a critical determinant of isoflavone productivity in hairy root cultures of *Lotus japonicas*”, *Plant and Cell Physiology*, vol. 48, pp. 1652–1657, 2007.

[166] J.E. Dotzlaf, and W.K. Yeh, "Copurification and characterization of deacetoxycephalosporin C synthetase/hydroxylase from *Cephalosporium acremonium*", *Journal of Bacteriology*, vol. 169, pp. 1611-1618, 1987.

[167] J.E. Baldwin, R.M. Adlington, R.T. Aplin, N.P. Crouch, and R. Wilkinson, "Cephalosporin C biosynthesis: On the mechanism of hydroxylation of deacetoxycephalosporin C to deacetylcephalosporin C", *Tetrahedron*, vol. 48 (no. 33), pp. 6853-6862, 1992.

[168] Q. Zhang, N. Shirley, J. Lahnstein, and G.B. Fincher, "Characterization and expression patterns of UDP-D-glucuronate decarboxylase genes in barley", *Plant Physiology*, vol. 138, pp. 131-141, 2005.

[169] K. Suzuki, Y. Suzuki, and S. Kitamura, "Cloning and expression of a UDP-glucuronic acid decarboxylase gene in rice", *Journal of Experimental Botany*, doi: 10.1093/jxb/erg203, 2003.

[170] H.P. Mock, and B. Grimm, "Reduction of uroporphyrinogen decarboxylase by antisense RNA expression affects activities of other enzymes involved in tetrapyrrole biosynthesis and leads to light-dependent necrosis", *Plant Physiology*, vol. 113, pp. 1101-1112, 1997.

[171] Mock HP, Keetman U, Kruse E, Rank B, and Grimm B, "Defense responses to tetrapyrrole-induced oxidative stress in transgenic plants with reduced uroporphyrinogen decarboxylase or coproporphyrinogen oxidase activity", *Plant Physiology*, vol. 116, pp. 107-116, 1998.

[172] J.Y. Han, J.G. In, Y.S. Kwon, and Y.E. Choi, "Regulation of ginsenoside and phytosterol biosynthesis by RNA interferences of squalene epoxidase gene in *Panax ginseng*," *Phytochemistry*", vol. 71, pp. 36-46, 2010.

[173] F. He, Y. Zhu, M. He, and Y. Zhang, "Molecular cloning and characterization of the gene encoding squalene epoxidase in *Panax notoginseng*", *DNA Sequence*, vol. 19 (no. 3), pp. 270-273, 2008.

[174] A.C.A. Yendo, F. de Costa, G. Gosmann, and A.G. Fett-Neto, "Production of plant bioactive triterpenoid saponins: Elicitation strategies and target genes to improve yields", *Molecular Biotechnology*, vol. 46, pp. 94-104, 2010.

[175] I.J. Flores-Sánchez, J. Ortega-López, M. del Carmen Montes-Horcasitas, and A.C. Ramos-Valdivia, "Biosynthesis of sterols and triterpenes in cell suspension cultures of *Uncaria tomentosa*", *Plant and Cell Physiology*, vol. 43, pp. 1502-1509, 2002.

[176] S. Razdan, W.W. Bhat, S. Rana, N. Dhar, S.K. Lattoo, R.S. Dhar, and R.A. Vishwakarma, "Molecular characterization and promoter analysis of squalene epoxidase gene from *Withania somnifera* (L.) Dunal", Molecular Biology Reports, vol. 40, pp. 905-916, 2013.

[177] T. Nakayama, H. Suzuki, and T. Nishino, "Anthocyanin acyltransferases: specificities, mechanism, phylogenetics, and applications", *Journal of Molecular Catalysis B: Enzymatic*, vol. 23, pp. 117-132, 2003.

[178] H. Fujiwara, Y. Tanaka, Y. Fukui, M. Nakao, T. Ashikari, and T. Kusumi, "Anthocyanin 5-aromatic acyltransferase from *Gentiana triflora*. Purification, characterization and its role in anthocyanin biosynthesis", *European Journal of Biochemistry,* vol. 249, pp. 45-51, 1997.

[179] W.M. Atkins, "Non-Michaelis-Menten kinetics in cytochrome P450-catalyzed reactions", *Annual Review of Pharmacology and Toxicology,* vol. 45, pp. 291-310, 2005.

[180] Y. Kapelyukh,1 M.J.I. Paine, J.D. Marechal, M.J. Sutcliffe, C.R. Wolf, and G.C.K. Roberts, "Multiple substrate binding by cytochrome P450 3A4: Estimation of the number of bound substrate molecules", *Drug Metabolism and Disposition*, vol. 36, pp. 2136-2144, 2008.

[181] C.A. Peebles, G.W. Sander, E.H. Hughes, R. Peacock, J.V. Shanks, and K.Y. San, "The expression of 1-deoxy-D-xylulose synthase and geraniol-10-hydroxylase or anthranilate synthase increases terpenoidindole alkaloid accumulation in *Catharanthus roseus* hairy roots", Metabolic Engineering, vol. 13, pp. 234-240, 2011.

[182] N. Gupta, S.K. Sharma, J.C. Rana and R.S. Chauhan "Expression of flavonoid biosynthesis genes vis-à-vis rutin content variation in different growth stages of *Fagopyrum* species", *Journal of Plant Physiology*, vol. 168, 2117-2123, 2011.

[183] M.B. Ali, and D.H. McNear, "Induced transcriptional profiling of phenylpropanoid pathway genes increased flavonoid and lignin content in *Arabidopsis* leaves in response to microbial products", *BMC Plant Biology*, vol. 14, pp. 84, 2014.

[184] W. Liu, M. Chen, C. Yang, Y. Yang, X. Lan, and Z. Liao, "A new 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase gene from *Taxus media*: Cloning, characterization and functional complementation", Journal of Medicinal Plants Research, vol. 3, pp. 395-402, 2009.

[185] K. Kumar, S.R. Kumar, V. Dwivedi, A. Rai, A.K. Shukla, K. Shanker, D.A. Nagegowda, "Precursor feeding studies and molecular characterization of geraniol synthase establish the limiting role of geraniol in monoterpene indole alkaloid biosynthesis in *Catharanthus roseus* leaves", *Plant Science*, http://dx.doi.org/10.1016/j.plantsci.2015.07.007, 2015.

[186] L. Cui, X. Ni, Q. Ji, et al., "Co-overexpression of geraniol-10-hydroxylase and strictosidine synthase improves anti-cancer drug camptothecin accumulation in *Ophiorrhiza pumila*", *Scientific Reports*, vol. 5, pp. 8227, 2015.

[187] Q. Pan, Q. Wang, F. Yuan, et al., "Overexpression of ORCA3 and G10H in *Catharanthus roseus* plants regulated alkaloid biosynthesis and metabolism revealed by NMR-metabolomics", *PLoS One*, vol. 7 (no. 8), pp. e43038, 2012.

[188] H.J. Klee, Y.M. Muskopf, and C.S. Gasser, "Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphatesynthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants", *Molecular Genetics and Genomics*, vol. 210, pp. 437-442, 1987.

[189] K.M. Herrmann, "The shikimate pathway: early steps in the biosynthesis of aromatic compounds", *The Plant Cell*, vol. 7, pp. 907-919, 1995.

[190] N. Bauer, H. Fulgosi, and S. Jelaska, "Over expression of phenylalanine ammonia-lyase in transgenic roots of *Coleus blumei* alters growth and rosmarinic acid synthesis", *Food Technology and Biotechnology*, vol. 49, pp. 24–31, 2011.

[191] L.M. Lois, M. Rodríguez-Concepción, F. Gallego, N. Campos, and A. Boronat, "Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase", *The Plant Journal*, vol. 22, pp. 503–513, 2000.

[192] J.M. Estévez, A. Cantero, A. Reindl, S. Reichler, and P. León, "1-deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidicisoprenoid biosynthesis in plants", *Journal of Biological Chemistry*, vol. 276, pp. 22901–22909, 2001.

[193] J. Battilana, F. Emanuelli, G. Gambino, I. Gribaudo, F. Gasperi, P.K. Boss, M.S. Grando, "Functional effect of grapewine 1-deoxy-D-xylulose 5-phosphate synthase substitution K284N on Muscat flavour formation", *Journal of Experimental Botany*, vol. 62, pp. 5497-5508, 2011.

[194] W. Hua, P. Zheng, Y. He, L. Cui, W. Kong, and Z. Wang, "An insight into the genes involved in secoiridoid biosynthesis in *Gentiana macrophylla* by RNA-seq", *Molecular Biology Reports*, doi: 10.1007/s11033-014-3352-x, 2014.

[195] G.N. Ye, P.T. Hajdukiewicz, D. Broyles, D. Rodriguez, C.W. Xu, N. Nehra, and J.M. Staub, "Plastid-expressed 5-enolpyruvyl shikimate-3-phosphatesynthase genes provide high level glyphosate tolerance in tobacco", The Plant Journal, vol. 25, pp. 261-270, 2001.

[196] G. Cao, Y. Liu, S. Zhang, et al., "A novel 5-enolpyruvylshikimate-3-phosphate synthase shows high glyphosate tolerance in *Escherichia coli* and tobacco plants", *PLoS One*, vol. 7, pp. e38718, 2012.

[197] B. Garg, N. Vaid, and N. Tuteja, "In-silico analysis and expression profiling implicate diverse role of EPSPS family genes in regulating developmental and metabolic processes", *BMC Research Notes*, vol. 7, pp. 58, 2014.

[198] M.C. Cabe, and C. Gopalan, "Transformation of tobacco (*Nicotiana tabaccum*) with ESPS (5-enolpyruvlshikimate 3-phosphatesynthase)-a key enzyme in the shikimate pathway", SACNAS National Conference, Los Angeles Convention Center, October 16-18, 2014

[199] G. Kai, H. Xu, C. Zhou, et al., "Metabolic engineering tanshinone biosynthetic pathway in *Salvia miltiorrhiza* hairy root cultures", *Metabolic Engineering*, vol. 13, pp. 319-327, 2011.

[200] P. Alam, and M.Z. Abdin, "Over-expression of HMG-CoA reductase and amorpha-4,11-diene synthase genes in *Artemisia annua* L. and its influence on artemisinin content", *Plant Cell Reports*, vol. 30, pp. 1919-1928, 2011.

[201] Q. Shen, Y.F. Chen, T. Wang, et al., "Over expression of the cytochrome P450 monooxygenase (cyp71av1) and cytochrome P450 reductase (cpr) genes increased artemisinin content in *Artemisia annua* (Asteraceae)", *Genetics and Molecular Research*, vol. 11, 3298-3309, 2012.

[202] M. Fujisawa, E. Takita, H. Harada, et al., "Pathway engineering of *Brassica napus* seeds using multiple key enzyme genes involved in keto carotenoid formation", *Journal of Experimental Botany*, vol. 60 (no. 4), 1319-1332, 2009.

[203] Y.M. Kang, D.J. Park, D.G. Lee, et al., "Over expression of IPP isomerase and limonene synthase enzymes in *Mentha spicata* and their influence on the terpenoid metabolism", *Romanian Biotechnological Letters*, vol. 20 (no. 2), pp. 10358-10368, 2015.

[204] R.B. Croteau, E.M. Davis, K.L.Ringer, and M.R. Wildung, "(-)-Menthol biosynthesis and molecular genetics", *Naturwissenschaften*, vol. 92, pp. 562–577, 2005.

[205] B.J. Brecke, and W.B. Duke, “Effect of glyphosate on intact bean plants (*Phaseolus vulgaris* L.) and isolated cells”, *Plant Physiology*, vol. 66, pp. 656-659, 1980.

[206] R.E. Hoagland, “Effects of glyphosate on metabolism of phenolic compounds: VI. Effects of glyphosine and glyphosate metabolites on phenylalanine ammonia-lyase activity, growth, and protein, chlorophyll, and anthocyanin levels in Soybean (*Glycine max*) seedlings”, *Weed Science*, vol. 28, pp. 393-400, 1980.

[207] G. Peiser, G. Lopez-Galvez, M. Cantwell, and M.E. Saltveit, “Phenylalanine ammonia lyase inhibitors control browning of cut lettuce”, *Postharvest Biology and Technology*, vol. 14, pp. 171-177, 1998.

[208] T. Kamo, N. Hirai, M. Tsuda, and D. Fujioka, “Changes in the content and biosynthesis of phytoalexins in banana fruit”, *Bioscience Biotechnology and Biochemistry*, vol. 64, pp. 2089-2098, 2000.

[209] A.W. Alberts, J. Chen, G. Kuron, et al., “Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent”, *Proceedings of the National Academy of Science of the United States of America*, vol. 77, pp. 3957-3961, 1980.

[210] J. Schwender, C. Mueller, J. Zeidler, and H.K. Lichtenthaler, “Cloning and heterologous expression of a cDNA encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *Arabidopsis thaliana*”, *FEBS Letters*, vol. 455, pp. 140-144, 1999.

[211] F. Chen, D. Tholl, J. Bohlmann, and E. Pichersky, “The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom”, *The Plant Journal*, vol. 66, pp. 212-229, 2011.

[212] W. Eisenreich, S. Sagner, M.H. Zenk, and A. Bacher, “Monoterpene essential oils are not of mevalonoid origin”, *Tetrahedron Letters*, vol. 38, pp. 3889-3892, 1997.

[213] A. Hemmerlin, J.F. Hoeffler, O. Meyer, et al., “Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells”, *Journal of Biological Chemistry*, vol. 278, pp. 26666-26676, 2003.

[214] M.L. Healy-Fried, T. Funke, M.A. Priestman, H. Han, and E. Schönburnn, “Structural basis of glyphosate tolerance resulting from mutations of Pro101 in *E. coli* EPSP synthase”, *Journal of Biological Chemistry*, vol. 282, pp. 32949-32955, 2007.

[215] H. Hollander, and N. Amrhein "The site of the inhibition of the shikimate pathway by glyphosate: I. Inhibition by glyphosate of phenylpropanoid synthesis in buckwheat (*Fagopyrum esculentum* moench)," *Plant Physiology*, vol. 66, pp. 823-829, 1980.

[216] D. Chakraborty, D. Sircar, and A. Mitra, "Phenylalanine ammonia-lyase-mediated biosynthesis of 2-hydroxy-4-methoxybenzaldehyde in roots of *Hemidesmus indicus*", *Journal of Plant Physiology*, vol. 165, pp. 1033-1040, 2008.

[217] S. Zhao, L. Wang, L. Liu, Y. Liang, Y. Sun, and J. Wu, "Both the mevalonate and the non-mevalonate pathways are involved in ginsenoside biosynthesis", *Plant Cell Reports*, vol. 33, pp. 393-400, 2014.

[218] O. Laule, A. Furholz, H. Chang, et al., "Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*", *Proceedings of the National Academy of Science of the United States of America*, vol. 100, pp. 6866-6871, 2003.

[219] M.A. Hussain, and Y.S. Bedi, "Authentication of *Picrorhiza kurrooa* Royle ex Benth. using DNA fingerprint", *International Journal of Agricultural Sciences*, vol. 2 (no. 6), pp. 511-521, 2012.

[220] Y. Gong, S. Xu, W. Mao, Q. Hu, G. Zhang, J. Ding, and L. Ya-dan, "Developing new SSR markers from ESTs of pea (*Pisum sativum* L.)", *Journal of Zhejiang University Science B*, vol. 11 (no. 9), pp. 702-707, 2010.

[221] L. Cardle, L. Ramsay, D. Milbourne, M. Macaulay, D. Marshall, and R. Waugh, "Computational and experimental characterization of physically clustered simple sequence repeats in plants", *Genetics*, vol. 156, pp. 847–854, 2000.

[222] P.K. Gupta, S. Rustgi, S. Sharma, R. Singh, N. Kumar, and HS Balyan, "Transferable EST-SSR markers for the study of polymorphism and genetic diversity in bread wheat", *Molecular Genetics and Genomics*, vol. 270 (no. 4), pp. 315-323, 2003.

[223] D. Metzgar, J. Bytof, and C. Wills, "Selection against frameshift mutations limits microsatellite expansion in coding DNA", *Genome Research*, vol. 10 (no. 1), pp. 72–80, 2000.

[224] M.V. Katti, P.K. Ranjekar, and V.S. Gupta, "Differential distribution of simple sequence repeats in eukaryotic genome sequences", *Molecular Biology and Evolution*, vol. 18, pp. 1161-1167, 2001.

[225] Y.C. Li, A.B. Korol, T. Fahima, and E. Nevo, "Microsatellites within genes: structure, function, and evolution", *Molecular Biology and Evolution*, vol. 21, pp. 991-1007, 2004.

[226] L. Zhang, D. Yuan, S. Yu, Z. Li, and Y. Cao, "Preference of simple sequence repeats in coding and non-coding regions of *Arabidopsis thaliana*", *Bioinformatics*, vol. 20, pp. 1081-1086, 2004.

[227] A. Gadaleta, A. Giancasproa, S. Zacheoa, D. Nigroa, S.L. Giovea, P. Colasuonnoa, and A. Blancoa, "Comparison of genomic and EST-derived SSR markers in phylogenetic analysis of wheat", *Plant Genetic Resources*, vol. 9 (no. 2), pp. 243-246, 2011.

[228] J. D. Wren, E. Forgacs, J. W. Fondon 3rd, et al., "Repeat polymorphisms within gene regions: phenotypic and evolutionary implications", *American Journal of Human Genetics*, vol. 67, pp. 345-356, 2000.

[229] E.N. Trifonov, "Tuning function of tandemly repeating sequences: a molecular device for fast adaptation", in *Evolutionary theory and processes: Modern horizons*, S.P. Wasser (ed.), Kluwer Academic Publishers. Amsterdam, Netherlands, 2003, pp. 1-24.

[230] K.U. Torii, "Leucine-rich repeat receptor kinases in plants: structure, function, and signal transduction pathways", *International Review of Cytology*, vol. 234, pp. 1-46, 2004.

[231] P.J. Rushton, I.E. Somssich, P. Ringler, and Q.J. Shen, "WRKY transcription factors", *Trends in Plant Science*, vol. 15 (no. 5), pp. 247-58, 2010.

[232] S.P. Pandey, and I.E. Somssich, "The Role of WRKY Transcription Factors in Plant Immunity", *Plant Physiology*, vol. 150 (no. 4), pp. 1648-1655, 2009.

[233] L. Wang, C. Xu, C. Wang, and Y. Wang, "Characterization of a eukaryotic translation initiation factor 5A homolog from *Tamarix androssowii* involved in plant abiotic stress tolerance" *BMC Plant Biology*, vol. 12, pp. 118, 2012.

[234] D.T. Dennis, and T.P. Coultate, "Phosphofructokinase, a regulatory enzyme in plants", *Biochemical and Biophysical Research Communications*, vol. 25 (no. 2), pp. 187-191, 1966.

[235] Y. Huang, C. Zhang, and D. Li, "Low genetic diversity and high genetic differentiation in the critically endangered *Omphalogramma souliei* (Primulaceae): implications for its conservation", *Journal of Systematics and Evolution*, vol. 47 (no. 2), pp. 103-109, 2009.

[236] Y. Qiu, X. Zhou, C. Fu, and Y.G. Chan. "A preliminary study of genetic variation in the endangered, Chinese endemic species *Dysosma versipellis* (Berberidaceae)", *Botanical Bulletin- Academia Sinica Taipei,* vol. 46, pp. 61-69, 2005.

[237] L.I. Fagen, and X.I.A. Nianhe, "Population structure and genetic diversity of an endangered species, *Glyptostrobus pensilis* (Cupressaceae). *Botanical Bulletin-Academia Sinica Taipei,* vol. 46, pp. 155-162, 2005.

[238] J.L. Hamrick, and M.J.W. Godt, "Allozyme diversity in plant species", in *Plant population genetics, breeding, and genetic resources*, A.H.D. Brown, M.T. Clegg, A.L. Kahler, and B.S. Weir (eds.), Sinauer, Sunderland, 1989, pp. 43-63.

[239] R. Frankham, "Conservation Genetics", *Annual Review of Genetics*, vol. 29, pp. 305-327, 1995.

[240] Y.G. Cho, T. Ishii, S. Temnykh, et al., "Diversity of microsatellites derived from genomic libraries and GenBank sequences in rice (*Oryza sativa* L.)", *Theoretical and Applied Genetics*, vol. 100, pp. 713-722, 2000.

[241] K. Chabane, G.A. Ablett, G.M. Cordeiro, J. Valkoun, and R.J. Henry, "EST versus genomic derived microsatellite markers for genotyping wild and cultivated barley", *Genetic Resources and Crop Evolution*, vol. 52, pp. 903-909, 2005.

[242] J. Hu, L. Wang, and J. Li, "Comparison of genomic SSR and EST-SSR markers for estimating genetic diversity in cucumber", *Biologia Plantarum*, vol. 55 (no. 3), pp. 577-580, 2010.

[243] N. Tabbasam, Y. Zafar, and Mehboob-ur-Rahman, "Pros and cons of using genomic SSRs and EST-SSRs for resolving phylogeny of the genus *Gossypium*", *Plant Systematics and Evolution*, vol. 300, pp. 559-575, 2013.

[244] V. Verma, A. Smelcerovic, S. Zuehlke, et al., "Phenolic constituents and genetic profile of *Hypericum perforatum* L. from India", *Biochemical Systematics and Ecology*, vol. 36, pp. 201-206.

LIST OF PUBLICATIONS

Research publications

[1] **Shitiz K**, Pandit S, Chauhan RS, Sood H (2013). Picrosides content in the rhizomes of *Picrorhiza kurroa* Royle ex Benth traded for herbal drugs in the markets of north India. *International Journal of Medicinal and Aromatic Plants* 3(2):226-233

[2] **Shitiz K**, Pandit S, Chanumolu S, Sood H, Singh H, Singh J, Chauhan RS (2015). Mining simple sequence repeats in *Picrorhiza kurroa* transcriptomes for assessing genetic diversity among accessions varying for picrosides contents. *Plant Genetic Resources*. doi:10.1017/S1479262115000404

[3] **Shitiz K**, Sharma N, Pal T, Sood H, Chauhan RS. NGS transcriptomes and enzyme inhibitors unravel complexity of picrosides biosynthesis in *Picrorhiza kurroa* Royle ex. Benth. *PLoS One* (Communicated, 2015)

[4] Pandit S, **Shitiz K**, Sood H, Chauhan RS (2012). Differential biosynthesis and accumulation of picrosides in an endangered medicinal herb *Picrorhiza kurroa***.** *Journal of Plant Biochemistry and Biotechnology* 22:335-42

[5] Pandit S, **Shitiz K**, Naik PK, Sood H, Chauhan RS (2013). Expression pattern of fifteen genes of non-mevalonate (MEP) and mevalonate (MVA) pathways in different tissues of endangered medicinal herb *Picrorhiza kurroa* with respect to picrosides content. *Molecular Biology Reports* 40:1053-63

[6] Sharma N, **Shitiz K**, Kalia D, Ahuja JK, Chauhan RS, Sood H (2014). Expression analysis of key genes for picroside-I production in *in vitro* grown shoots of different genotypes and species of endangered herb *Picrorhiza* spp. *International Journal of Basic and Applied Biology* 2:36-39

[7] Kumar V, **Shitiz K**, Chauhan RS, Sood H, Tandon C (2015). Tracking dynamics of enzyme activities and their gene expression in *Picrorhiza kurroa* with respect to picroside accumulation. *Journal of Plant Biochemistry and Biotechnology*. doi:10.1007/s13562-015-0317-7

[8] Kumar V, Sharma N, **Shitiz K**, Singh TR, Tandon C, Sood H, Chauhan RS (2015). An insight into conflux of metabolic traffic leading to picroside I biosynthesis by tracking molecular time course changes in a medicinal herb, *Picrorhiza kurroa*. *Plant Cell Tissue and Organ Culture*. doi:10.1007/s11240-015-0839-7

Conference publications

[1] **Shitiz K**, Kumar V, Pal T, Sood H, Chauhan RS. Transcript profiles of phenylpropanoid pathway genes vis-à-vis picrosides content in an endangered medicinal herb *Picrorhiza kurroa* Royle ex. Benth. *Proceedings of the International Conference on Emerging Trends in Biotechnology*, Jawaharlal Nehru University, New Delhi, November 6-9, 2014

[2] **Shitiz K**, Sood H, Chauhan RS. Mining simple sequence repeats (SSRs) in *Picrorhiza kurroa* transcriptomes and assessment of genetic diversity vis-a-vis picrosides content. *Proceedings of the National Symposium on Advances in Biotechnology for Crop Improvement*, Eternal University, Baru Sahib (H.P.), July 12, 2014

[3] Pandit S, **Shitiz K**, Chauhan RS, Sood H. Growth and Developmental Status of an Endangered Medicinal Herb (*Picrorhiza kurroa*) Influences Differential Biosynthesis and Accumulation of Major Chemical Constituents (Picroside-I and Picroside-II). *Proceedings of the International conference in biochemistry, biosciences and biotechnology*, World Academy of Science Engineering and Technology, Venice (Italy), April 11 -13, 2012

Patent filed

[1] Kumar V, Sood H, Pandit S, **Shitiz K**, Chauhan RS (2012). Proposed biosynthetic pathway for the production of Picroside-I and Picroside-II and their intermediates in *Picrorhiza kurroa* (Patent Application No. 1922/DEL/2012)