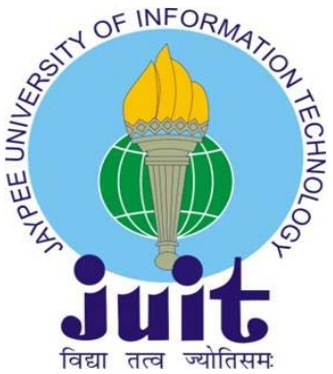
DIFFERENTIAL PROTEOMIC ANALYSIS FOR IDENTIFYING PROTEINS INVOLVED IN THE BIOSYNTHESIS OF PICROSIDE−I AND PICROSIDE−II FROM *Picrorhiza kurroa*−AN ENDANGERED MEDICINAL HERB

Thesis submitted in fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

AMIT SUD



Department of Biotechnology and Bioinformatics

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

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DECLARATION

I hereby declare that the work reported in the Ph.D. thesis entitled **"Differential proteomic analysis for identifying proteins involved in the biosynthesis of picroside−I and picroside−II from *Picrorhiza kurroa*−An endangered medicinal herb"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic of my work carried out under the supervision of **Dr. Hemant Sood** and co-supervision of **Dr. Chanderdeep Tandon**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. Thesis

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CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Differential proteomic analysis for identifying proteins involved in the biosynthesis of picroside−I and picroside−II from *Picrorhiza kurroa*−An endangered medicinal herb"** which is being submitted by **Amit Sud (Enrollment No. 106567)** in fulfillment for the award of degree of **Doctor of Philosophy** in **Biotechnology** at **Jaypee University of Information Technology, Waknaghat, India** is a bonafide record of his original work carried out 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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Dedicated to my loving Parents

&

Brothers for their endless support

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Amit Sud

ABSTRACT

Picrorhiza kurroa (*P. kurroa*), a medicinally vital herb found principally in the North-Western Himalayan region has several medicinal properties connected with it because of active ingredients like picroside-I and picroside-II. Due to gigantic medicinal applications associated with this herb, it's overexploitation from its natural habitat has caused in it being registered as a threatened species in the Red Data Book. The proteins accompanying picroside biosynthesis and other phenotypical characters of *P. kurroa* remained elusive. Thus, evolving any strategy for boosting the production of picrosides would necessitate awareness on the proteins allied with the biosynthesis of these two active compounds. An earlier study by Sood and Chauhan, 2011 has revealed that tissue cultured *P. kurroa* kept at 15 ± 1 °C show heightened picroside-I content and shoot biomass as compared to *P. kurroa* kept at 25 ± 1 °C. Discovering the proteins answerable for these two traits could deliver us with appropriate candidates for overexpression ensuing in a greater picroside content and shoot biomass the two imperative traits of economic importance. Also, stolons have been found to comprise both picroside-I and picroside-II in respectable quantities but is it only acting as a storage organ or is it also involved in their biosynthesis endured to be investigated. The current study, thus, explored (i) Optimization of protein extraction from different parts of *P. kurroa* (shoots, roots and stolons); (ii) Differential protein expression studies in *P. kurroa* to identify proteins involved in the biosynthesis of picroside-I and increased shoot biomass; and (iii) Differential protein expression studies in *P. kurroa* to investigate the presence of proteins involved in the biosynthesis of picroside-I and picroside-II in stolons.

Assessment of literature revealed that no information was existing on the proteome analysis of *P. kurroa* w.r.t. picroside−I and II biosynthesis. Therefore, the first objective was to optimize protein extraction from different tissues of *P. kurroa* such as shoots, roots, and stolons. Of the four extraction methods (Tris-Cl buffer, phosphate buffer, TCA-acetone, and water) employed, Tris-Cl method succeeded in isolating the maximum concentration of proteins from shoots and roots of *P. kurroa* while TCA-Acetone was successful in extracting maximum concentration of proteins from the stolons of *P. kurroa*.

Conferring to a projected strategy differential proteomics analysis between two opposing conditions such as metabolite accumulation and non-accumulation could help in identifying the proteins accountable for the observed differences. Consequently, to identify proteins

connected with enhanced picroside-I levels and biomass in 15 ± 1 °C *P. kurroa* shoots as compared to 25 ± 1 °C *P. kurroa* shoots these two tissues were designated for comparative proteomics analysis. It has been experimentally authenticated that picroside-I content is ~17 times higher in 15 ± 1 °C shoots (2.03 mg/g fresh shoot wt.) as compared to 25 ± 1 °C shoots (0.12 mg/g fresh shoot wt.) and the shoot biomass is approximately 2 times more in 15 ± 1 °C (3.73 ± 0.5 g) as compared to 25 ± 1 °C (2.03 ± 0.3 g). Using SDS-PAGE and 2-D gel electrophoresis coupled with mass spectrometry-based protein identification revealed 108 altered proteins belonging to several functional categories like metabolic pathways, stress response, signalling pathways, photosynthesis, cell cycle, transport, transcription and translation factors and energy metabolism. Identification of certain enzymes of the picroside biosynthetic pathway that were foretold based on transcriptomic data provided extra support to the contribution of those enzymes in picroside biosynthesis. The study also revealed an intricate relationship amid various primary metabolic pathways and picroside biosynthetic pathway wherein primary metabolic pathways act as feeder pathways for feeding key metabolites into the picroside biosynthetic pathway. To explore the presence of biosynthetic enzymes in stolons comparative proteomic analysis was performed between stolons which contain both picroside-I and II and roots obtained from tissue cultured *P. kurroa* maintained at 15 ± 1 °C which contains neither picroside-I nor picroside-II. SDS-PAGE analysis coupled with mass spectrometric-based identification of differentially expressed proteins recognized certain metabolism-related proteins in stolons signifying their possible role in picroside biosynthesis.

This study highlights the importance of identifying proteins associated with picroside biosynthesis, increased shoot biomass and gives a perception into how the protein profile vicissitudes when *P. kurroa* is exposed to stress (low temperature) empowering it to survive with the stressful state. Increasing the expression of genes coding for proteins associated with picroside biosynthesis and increased shoot biomass could upshot in enhanced picroside production and shoot biomass the latter also ensuing in enhanced picroside-I content as more picroside-I containing biomass would be accessible per plant.

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LIST OF ACRONYMS & ABBREVIATIONS

10HD	10-hydroxygeraniol dehydrogenase
2HFD	2-hydroxyisoflavanone dehydratase
AACT	Acetoacetyl-CoA thiolase
ACT	Anthocyanin acyltransferase
ADH	Aldehyde dehydrogenase
AGPAT	1-acyl-sn-glycerol-3-phosphate acyltransferase
APD	Arogenate/prephenate dehydratase
APS	Ammonium Persulfate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CAM	Caffeic acid-3-O-methyltransferase
CDP-ME	4-diphosphocytidyl-2-C-methylerythritol
CDP-ME2P	4-diphosphocytidyl-2-methyl-D-erythritol 2-phosphate
CM	Chorosmate mutase
CME-PP	2-C-methyl-D-erythritol 2,4-cyclopyrophosphate
CMP	Cytidine monophosphate
CPM	Cytochrome P-450 monooxygenase
CS	Chorismate synthase
CTP	Cytidine triphosphate
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
G10H	Geraniol 10-hydroxylase
GDPS	Geraniol diphosphate synthase

GPP	Geranyl pyrophosphate
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
IDI	Isopentenyl diphosphate isomerase
IS	Iridoid Synthase
IPP	Isopentenyl pyrophosphate
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
LC/ESI MS	Liquid Chromatography/Electrospray ionization Mass Spectroscopy
MALDI-TOF	Matrix-assisted laser desorption ionization Time of flight
MC	Monoterpene Cyclase
MECPS	2-C-methylerythritol-2,4-cyclophosphate synthase
MEP	2-C-methyl-D-erythritol 4-phosphate
MVDD	Mevalonate diphosphate decarboxylase
MK	Mevalonate kinase
MPK	Mevalonate-5-phosphate kinase
NBT	Nitroblue tetrazolium
NOX	NADPH oxidase activity
PAL	Phenylalanine ammonia lyase
PBST	Phosphate Buffered Saline Tween-20
PTOX	Podophyllotoxin
PVDF	Polyvinylidene fluoride
QSD	Quinate/shikimate dehydrogenase
RC/DC	Reducing agent compatible/Detergent compatible
SAM	S-adenosyl-L-methionine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase

SK	Shikimate kinase
SQM	Squalene monooxygenase
TAT	Tyrosine aminotransferase
TCA	Trichloroacetic acid
UGD	Uridine diphosphate glucuronic acid decarboxylase
UGT	Uridine diphosphate glycosyltransferase
UPD	Uroporphyrinogen decarboxylase
WIN1	Wax Inducer 1
WLS	Wild Life Sanctuary
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

CHAPTER 1

INTRODUCTION

The histories of Western orthodox medicine and herbal medicine are inseparably intertwined because until the nineteenth century, with the advent of synthetic chemistry, all drugs were procured from natural sources, mostly from plants [1]. People belonging to diverse cultures have been using medicinal plants as a source of medicine for maintaining good health throughout history [2, 3]. India, an immense repository of medicinal plants has around 45,000 plant species with most them concentrated in regions like Eastern Himalayas, Western Ghats and Andaman and the Nicobar Islands. Over 7,500 species of plants are being used as medicines by several ethnic communities of India [2, 4, 5].

Plants have become an important part of our everyday lifestyle. For several years, intensive studies have been carried out on various plant constituents and their nutritional value [6]. Medicinal plants have been recognized all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments. About 60 % of the world's population and 70 % of Indian rural population relies on alternative medicines for their primary health care needs [7].

In India, a number of recognized indigenous system of medicine viz., Ayurveda, Siddha, Unani, Homeopathy, Yoga, and Naturopathy are being employed for the well-being of people. The safety associated with these natural products make them an ideal choice among rural and urban Indian communities [8].

Plants synthesize a variety of metabolites which can be classified into two categories namely, primary metabolites: which are involved in essential metabolic processes of the plant and secondary metabolites: which are not involved in the fundamental life processes of plant but in a variety of other roles which help plants in their survival and reproduction [6, 9-11].

The medicinal properties of various medicinal plants can be attributed to these secondary metabolites. It has been generally observed that a particular medicinal property is inherent to a specific plant species or groups which is in congruence with the fact that taxonomically distinct plants possess a different combination of secondary metabolites [12].

A number of drugs being used in conventional medicine were originally obtained from plants. Vincristine, an anticancer drug is obtained from periwinkle *(Catharanthus roseus* Linn. G. Donn.) [13]. Salicylic acid, a precursor of aspirin was originally derived from white willow bark and the meadowsweet plant *(Filipendula ulmaria (*L.) Maxim.) [14]. The well-known antimalarial drugs Artemisinin and Quinine were derived from *Artemisia annua* L. and *Cinchona pubescens* Vahl bark plant, respectively [15]. Morphine, codeine, and paregoric, used in the treatment of diarrhea and pain relief were derived from the opium poppy *(Papaver somniferum* L.) [16]. Digitoxin, a cardiac glycoside derived from foxglove plant *(Digitalis purpurea* L.) has been in use since 1775 [17].

The recent resurgence of interest in herbal medicines at a global scale has resulted in an upsurge in their demand. The World Health Organization has estimated that the current demand for medicinal plants is approximately US $14 billion per year. The demand from medicinal plant-based raw material is increasing at a pace of 15 to 25 % annually and WHO estimates an increase of more than US $5 trillion by 2050 [18]. Although the demand for medicinal plants is going up, some of them are facing the constant threat of extinction under the pressure of enormous exploration and habitat degradation [2].

About 95% of the medicinal plants are acquired from their natural habitats. Numerous studies have revealed that the current methods of harvesting are the main reasons for depletion of resource bases. The harvesting practices that are being carried out by medicinal plant-based industries are inefficient, imperfect, informal and opportunistic resulting in the raw-material supply situation being unstable, unsustainable and exploitative. The absence of serious policy attention with environmental planning has resulted in a huge, enigmatic, and largely unregulated trade in medicinal plants, mainly from the wild, which continues to grow dramatically. Misperception also occurs in the identification of plant materials where the source of a specific drug is assigned to more than one plant, sometimes having vastly different morphological and taxonomical characters; therefore, adulteration is common in such cases [19, 20]. The medicinal and aromatic plants and their wild populations are facing serious threats owing to (1) the unimpeded and intensive collection on commercial scale, often concentrated in few areas, (2) the largely unmonitored trade, (3) destructive harvesting techniques, (4) trade structure variations in countries and (4) global habitat loss and alteration [21].

Presence of desired contents and a combination of major chemical constituents which in most of the case are secondary metabolites, modulate the quantity and efficacy of a herbal drug formulation.

Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups: the terpenoids, the alkaloids and the phenylpropanoid and allied phenolic compounds [22, 23]. Although, this represents only a fraction of what exists in nature but more than 36,000 terpenoids, 12,000 alkaloids, and 10,000 flavonoids have been discovered [24].

The developmental stage of the plant [25], as well as external stimuli [26], influence the biosynthesis and accumulation of medicinally important metabolites in a plant. The identification of these factors is crucial as they help in the uniform selection of plant material essential for the preparation of herbal drug formulation. Different batches of the same herbal ingredient may differ in quality due to a number of factors. Amount of active constituent generally vary between plant parts and it is not uncommon for herbal ingredients to be adulterated with parts of the plants not normally utilized [26]. The quality of herbal ingredients can be affected by (1) abiotic factors such as temperature, altitude, light, water and soil composition, (2) biotic factors such as plant interactions with microorganisms or plant physiological aspects, as phenology and ontogeny and (3) time scale variability which include conditions such as season, time of day, tissue ageand temperature [27]. Therefore, it becomes all the more important to specify the optimum time/condition for harvesting a plant as the level of secondary metabolite varies with a number of factors. Anthocyanins are reported to increase in response to salt stress [28]. In contrast to this, salt stress decreased anthocyanin level in the salt-sensitive species [29]. In tomato cultivars under salt stress, endogenous JA was found to accumulate [30]. [31] showed increased total phenolics content with the moderately saline level in red peppers. Drought often causes oxidative stress and was reported to show an increased amount of flavonoids and phenolic acids in willow leaves [32]. A reduction in chlorophyll content was reported in cotton under drought stress [33] and *Catharanthus roseus* [34]. Lower temperature resulted in increased accumulation of artemisinin in *Artemisia* spp. [35, 36], anthocyanin in *Nicotiana tabacum* [37] and *Malus* sp. [37]. *Matricaria chamomilla* has been shown to produce enhanced levels of flavonoids and phenolic acids at higher altitudes [38]. The accumulation of withanolide occurs in shoot tips and leaves of *Withania somnifera* [39]. The biosynthesis of rutin is influenced

by the growth and developmental stages of *Fagopyrum* species [40]. Increase in podophyllotoxin content was reported in the rhizomes of different age groups of *Podophyllum hexandrum Royle* [41]. The amount of berberine increased in the roots and rhizomes of 5-year-old *Berberis darwini* compared to 3-year-old plants [42]. Younger leaves were found to contain higher camptothecin (CPT) concentrations than older leaves. Within a branch, there was a linear decline in CPT concentration from leaves at the apex of the branch down to leaf no. 7. Comparing leaves of similar age, those from newer trees had elevated CPT concentrations than those from older trees [43]. As the above-mentioned factors are identified and optimized for a plant, they can prove to be of great help to farmers, herbal material collectors, researchers and herbal drug manufacturing industries in acquiring best quality plant material at a particular time when the amount of active constituent is at its maximum.

P. kurroa Royle ex. Benth also known as kutki or karu, is a perennial medicinal herb belonging to family Scrophulariaceae (Figure 1.1), predominantly found in the North-Western Himalayan regions at an altitude of 3000 – 5000 m [44, 45].

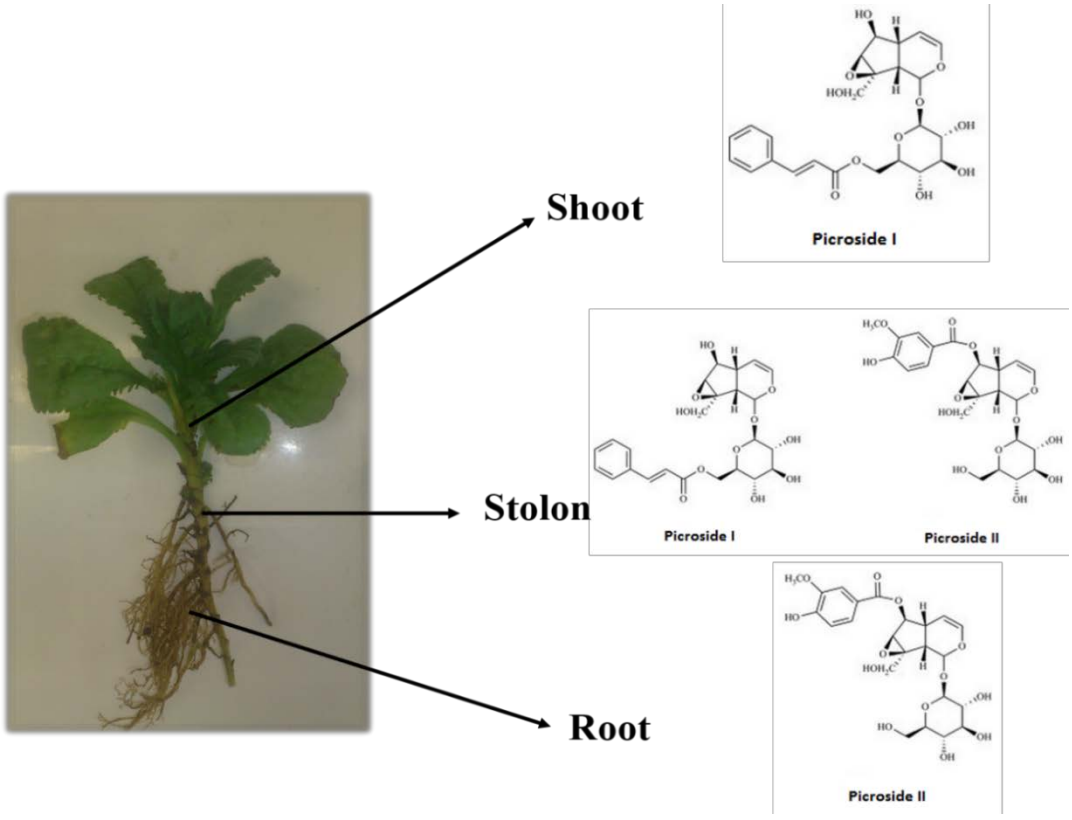


Figure 1.1 *Picrorhiza kurroa* plant with preferential accumulation of the two key metabolites picroside-I, and picroside-II in its different parts

P. kurroa in India, is primarily disseminated in the north-west, north-east, central and trans-Himalayan biogeographic zones [46, 47]. In Himachal Pradesh, the plant is distributed in Great Himalayan National Park, Dhauladhar WLS, Kugti WLS, regions of Lahaul-Spiti and alpine regions of Mandi, Kullu, Chamba, and Kinnaur districts [48, 49].

P. kurroa extracts are known to be hepatoprotective [50-54], antiallergic [53], antiasthmatic [53, 55], superoxide scavenging [50], anticarcinogenic [56], antioxidant [52, 53, 57], immunomodulatory [58], immunostimulant [59] and antidiabetic [60] and as such have been widely used for treating disorders like liver diseases, dyspepsia, chronic diarrhoea, and upper respiratory ailments [51].

A number of studies have been conducted for exploring the healing potential of *P. kurroa* especially its ability to heal hepatic injuries. *P. kurroa* showed the healing effect on rat livers after they had been subjected to hepatic injuries induced by various agents like ethanol [59], thioacetamide, galactosamine and carbon tetrachloride [61].

The medicinal properties of *P. kurroa* are attributed to the presence of iridoid glycosides such as picroside-I, picroside-II, picroside-III, picroside-IV, apocyanin, androsin, kutkoside, vermioside and specioside [51, 52].

A number of herbal formulations like Picroliv, Livokin, Livomap, Tefroliv etc. contain *P. kurroa* extracts rich in picroside-I and picroside-II content. The growing market demand of *P. kurroa* along with its inadequate cultivation and uncontrolled collection from the wild has resulted in it being listed as an endangered species in the Red Data Book. The plant is registered as vulnerable species in Red Data Book [62] and consequently in the 'endangered' species category, as per Conservation Assessment and Management Planning (CAMP) workshop [63]. Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed *P. kurroa* in its *annexure 11* that restricts its trade across nation's territory [64].

Standardized iridoids fraction of *P. kurroa,* called kutkin and Picroliv collectively form the term “Picrosides”. The crystallized iridoids fraction kutkin consists of the glycosides, P-I, and kutkoside in a ratio of 1:2 and other minor glycosides [57, 65]. Picroliv, alike kutkin is the less purified fraction, comprising of about 60% of an equal mixture of picroside I and kutkoside [66]. More recent publications described Picroliv as a standardized iridoid fraction containing 60% of this mixture in a 1:1.5 ratio [67].

Picroside-I (P-I) and picroside-II (P-II) the major picrosides differ from each other in terms of the attached functional group. In P-I, a cinnamoyl group is attached to the sugar moiety,

while in P-II vanilloyl group is attached to the 6th carbon of the iridoid ring (Figure 1.2) [68].

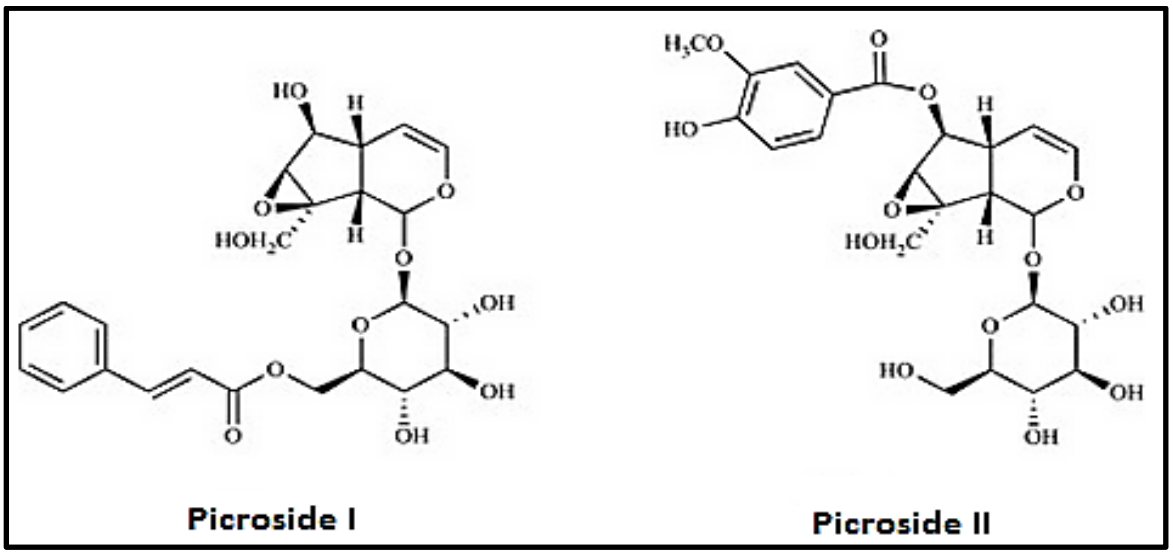


Figure 1.2 Chemical structure of Picroside I and Picroside II

Picroliv, containing primarily P-I and P-II, is reported to have hepatocurative effect against aflatoxin B1 induced hepatotoxicity in rats [69] and an immuno-stimulant activity against *Leishmania donovani* infection in hamsters [70].

Picroside-I and picroside-II accumulation in *P. kurroa* have been shown to occur differentially with picroside-I being formed primarily in the shoots and stolons and picroside-II being formed in roots and stolons [51, 53, 71]. Irrespective of the site of synthesis both P-I and P-II accumulate in rhizomes [53]. The biosynthesis and accumulation of P-I and P-II in *P. kurroa* at high altitudes and at a particular time of a season complicates the understanding of their biosynthetic biology. To gain an insight into their biosynthesis it becomes all the more essential to study the variations in P-I and P-II content synthesized/accumulated during different growth and developmental stages of *P. kurroa*. This knowledge would also prove useful to herbal industries in the optimization of commercial cultivation and production of uniform plant material.

Recently, the complete biosynthetic pathway of P-I and P-II biosynthesis (Figure 1.3) has been proposed [52, 72].

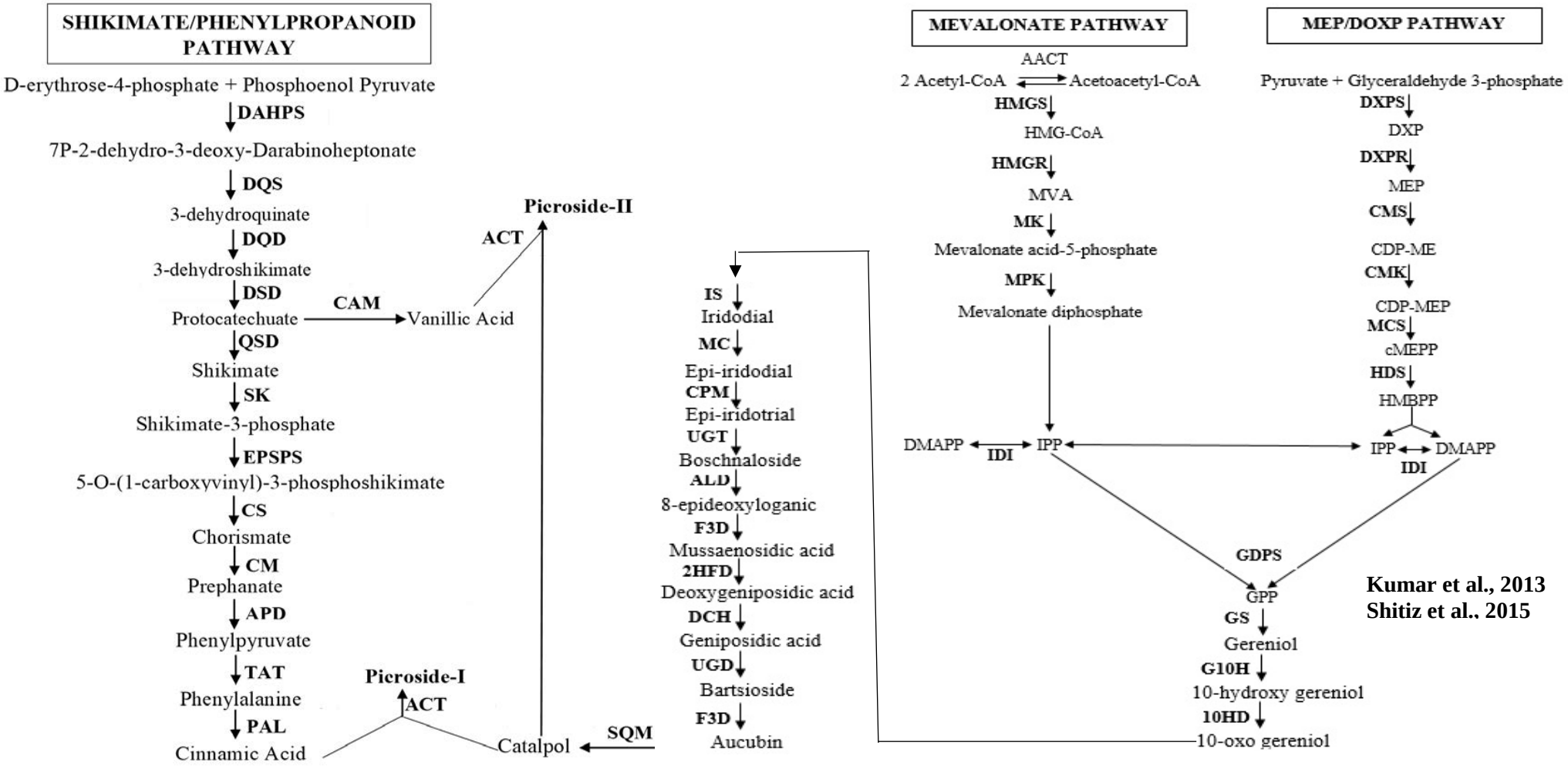


Figure 1.3 Amalgamation of the four biosynthetic pathways associated with picroside biosynthesis: MVA, MEP, iridoid and phenylpropanoid pathway

This pathway proposes that picrosides belonging to monoterpene family of secondary metabolites is derived from geranyl diphosphate (GDP) using the isoprenoid biosynthetic pathway. GDP is formed by the sequential head to tail addition of isopentenyl pyrophosphate (IPP) and its allelic isomer dimethylallyl pyrophosphate (DMAPP) through Mevalonate (MVA) and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. This GDP is then converted to catalpol via the iridoid pathway. If cinnamic acid from the phenylpropanoid pathway esterifies with catalpol it results in the formation of picroside-I and if vanillic acid esterifies with catalpol it results in picroside-II.

The simple C5 compounds IPP and DMAPP are the universal precursors of isoprenoids like geranyl, farnesyl, dolichols, sterols, ubiquinone, prenyl groups and isopentenylated tRNA's [73, 74]. The choice of a secondary metabolite being formed via the MVA pathway or the MEP pathway depends on the plant species under consideration. For example, natural rubber relies on MVA pathway, whereas stevioside is derived through MEP pathway [75].

For many years MVA pathway of isoprenoid biosynthesis was considered as the sole route for the production of IPP and DMAPP [76] until recently an alternate route to the biosynthesis of IPP and DMAPP known as the MEP or the non-mevalonate pathway was elucidated [77]. In the cytosol, IPP is generated by acetyl-CoA via the MVA pathway, which supplies precursor for the biosynthesis of sesquiterpenes [78]. In plastids, IPP for the biosynthesis of monoterpenes is produced via MEP pathway [79].

The MVA pathway is the source of IPP and DMAPP in most eukaryotes, archaea, a few eubacteria, the cytosol and mitochondria of plants, fungi and eukaryotic parasites *Trypanosoma* and *Leishmania* [80, 81]. The pathway initiates with the production of acetoacetyl-CoA from acetyl-CoA in a reaction catalyzed by thiolase. This acetoacetyl-CoA is then converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase followed by the conversion of HMG-CoA to MVA by HMG-CoA reductase. Next, MVA is phosphorylated to MVA 5-diphosphate firstly by mevalonate kinase (MVK) then by phosphomevalonate kinase (PMK). Decarboxylation by mevalonate diphosphate decarboxylase (MDD) results in IPP some of which is isomerized to DMAPP by an IPP isomerase.

The MEP pathway supplies IPP and DMAPP in plant chloroplasts, algae, cyanobacteria, eubacteria and apicomplexan parasites [77]. Characterization of this pathway is one of the best examples of modern proteomics research, driven by a combination of genomic

data and biological chemistry [76]. The pathway begins with the formation of 1-deoxy-D-xylulose 5-phosphate (DOXP) by the condensation of pyruvate and glyceraldehyde 3-phosphate catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS). This DOXP is then converted to MEP by 1-deoxy-D-xylulose-5-phosphate reductoisomerase followed by 4-diphosphocytidyl-2*C*-methyl-D-erythritol cytidylyltransferase catalyzing the conversion of MEP to 4-diphosphocytidyl-2*C*-methyl-D-erythritol (CDP-ME) using CTP. CDP-ME is then phosphorylated by the 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase to yield 4-diphosphocytidyl-2*C*-methyl-D-erythritol-2-phosphate (CDP-ME2P) and ADP. Next, 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate synthase catalyzes the conversion of CDP-ME2P to 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MECP) and CMP. The cyclodiphosphate MECP undergoes a two-electron reduction and elimination to form 1-hydroxy-2-methyl-2-butenyl-4-diphosphate (HMBPP) in a reaction catalyzed by 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase. Finally, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase catalyzes the production of IPP (85%) and some DMAPP (15%) from the pool of HMBPP.

Isopentenyl diphosphate isomerase catalyzes the interconversion of IPP and its allylic isomer DMAPP. Geranyl diphosphate synthase (GDPS), catalyzes the condensation of dimethylallyl diphosphate and isopentenyl diphosphate to geranyl diphosphate. GDP after cyclisation yields iridoid; the addition of glucose and cinnamate yields P-I, whereas addition of glucose and vanillate to this moiety yields P-II.

Due to the existence of cross-talks between two IPP biosynthetic pathways via an isopentenyl pyrophosphate (IPP) transporter believed to be located in the plastid envelope membrane the compartmentalization is not absolute as at least one of the metabolites can be exchanged [82, 83]. The species and the concentration of exogenous precursors influence the extent of crosstalk. Crosstalk is generally assumed to be of lesser extent (<1%) in intact plants under physiological conditions [84]. Higher extent of crosstalk was observed in plant cell cultures when supplemented with 1-deoxyxylulose or mevalonate. [84].

Identification of several upregulated genes of both MVA pathway and the MEP pathway showed that both pathways contribute to the formation of GPP.

The final step in the formation of picrosides is the esterification of catalpol derived from iridoid biosynthetic pathway with various aromatic acids obtained from phenylpropanoid pathway [52].

Although the metabolites of the pathway have been deciphered, the proteins involved in their biosynthesis still needs to be elucidated. According to a proposed strategy, metabolites; proteins and transcriptional profiling under two physiological states (for example, metabolites accumulating versus non-accumulating) can provide a novel approach for pathway elucidation in plants [85, 86]. Although transcriptomics analysis can provide a great wealth of knowledge about various biological processes, but it is incomplete without proteome analysis which aids in the improved understanding of events occurring inside a cell. As the central dogma dictates the transcription of DNA to mRNA and the translation of mRNA to proteins, one would expect to find a correlation between mRNA and protein abundances. But this is not true, research has found the correlation between mRNA and protein to be poor [87-89]. Three reasons have been presumed for this poor correlation; a) Significant difference in the half-lives of proteins, b) Inability to get a clear picture because of a significant amount of error and noise in both protein and mRNA experiments and c) Various complicated post-translational modifications [88]. It has been observed that DNA sequence and mRNA expression studies fail to provide information regarding protein post-translational modification, structure and protein-protein interactions. For performing various functions almost all proteins undergo post-translational modification, hence, it becomes essential to analyze the protein content so as to get a better understanding of the various physiological processes [90].

Another important reason which necessitates the study of P. *kurroa* proteins is that proteomic studies to date have been carried out primarily in model plants such as *Arabidopsis thaliana*, *Oryza sativa* (rice), *Populus trichocarpa* (black cottonwood), and *Vitis vinifera* (grapevine) for which fully sequenced genomes are available. Very few studies have been conducted in relation to the biosynthesis of secondary metabolites in medicinal plants especially with the focus of identifying new enzymes involved in secondary metabolism [91].

Review of the literature reveals that no proteomic data is available as of today associated with the biosynthesis of picroside-I and picroside-II in *P. kurroa*. Hence, this study was carried out with the objective of identifying proteins for the first time related to the biosynthesis of picrosides in *P. kurroa* using differential proteomics approach

exploiting the differential condition of metabolite accumulation and metabolite non-accumulation.

Understanding the medicinal importance of the endangered species *P. kurroa*, and that no proteomics study has been done on this plant, the following objectives were laid down for the present thesis:

- Optimization of protein extraction from different parts of *P. kurroa* (shoots, roots, and stolons)
- Differential protein expression studies in *P. kurroa* to identify proteins involved in the biosynthesis of picroside-I and increased shoot biomass.
- Differential protein expression studies in *P. kurroa* to investigate the presence of proteins involved in the biosynthesis of picroside-I and picroside-II in stolons.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Introduction

Regardless of all the advancement in synthetic chemistry and biotechnology, plants are still a crucial source of medicinal preparations [92]. From a historical perspective, the production of medicines and the pharmacologic treatment of diseases began with the use of herbs. Prior to 1800, when medicine entered the scientific age, traditional herbal medicine was the unquestioned foundation for all standard textbooks on pharmacology [93]. It is only with the advent of "medical science" that phytotherapy was relegated to the status of an alternative modality. From the historical perspective, however, it is unfitting to pigeonhole phytotherapy as a special or alternative branch of medication. Medicinal plants are an indispensable module of traditional medicinal systems. Original chronicles propose that herbal medicines have been used and recognized in Indian, Chinese, Egyptian, Greek, and Roman medicinal systems for about five thousand years. The transcriptions of conventional medicine systems in India include Rigveda, Atherveda, Charak Samhita and Sushruta Samhita. Folk drugs are also imperative foundations for the home-grown healthcare system. India has been recognized as a rich fountain of medicinal plants from ancient civilizations [94]. Research and segregation of bioactive elements from medicinal plants and there pharmacological screening can aid in finding innovative therapeutic drugs. It is projected that more than 6000 higher plant species are included in codified systems such as Ayurveda, Siddha, and Unani and in folk medicine of India [95]. India is on the verge of a herbal uprising due to its affluence of herbs and is talented to supply medicinal plant resources to encounter the growing comprehensive mandate. Medicinal plants are not only central for the healthcare system but also vital to lift the economy and can convey a significant part in fiscal development. Sundry treasured medicinal plants are on the edge of annihilation owing to dilapidation of biodiversity [96].

While in developed countries health providers have abridged their necessity on the Plant Kingdom, the mainstream evolving countries still depend on herbal medicines. Nevertheless, in a complete turn-around, modern science and Western medicine are

getting fascinated in the healing herbs once more [97, 98]. As a consequence of this new fondness of leading industrialized nations towards medicinal herbs, a wealth of technical information is now coming available, particularly from sophisticated laboratories who are analyzing herbal ingredients and their effects with the latest technologies.

The term "medicinal plants" has been defined in innumerable ways in the past. One definition implies that only when medicinal properties are proven by Western research should a species be labeled a "medicinal plant" [99]. On the contrary, another author defines a medicinal plant as a term which indicates merely a species known to beneficially modulate the physiology of sick mammals, and that has been used by people [100]. For present purposes the following definition seems to be apt: "*Medicinal plants are those that are commonly used in treating and preventing specific ailments and diseases, and that are generally considered to play a beneficial role in health care*" [92].

Medicinal plants are an important part of the global economy. Their demand is increasing swiftly in both developing as well as industrialized nations. For example, In Europe and North America, the association of "all-natural" tag with these products along with aggressive marketing has fuelled the customer interest in herbal remedies [101]. The global resurgence in traditional and alternative healthcare systems has grown at an impressive rate. The herbal drug market is a lucrative one and the world herbal trade is expected to reach USD 7 trillion by 2050 [102].

This upsurge in global interest has created an escalating legitimate and "illegal" trades in plant materials, many of which are being routinely moved around the world. Most of these samples are collected in a completely unregulated manner in developing countries. In India, about 70 % of the modern medicines are obtained from natural resources and several other synthetic analogs have been made from prototype compounds isolated from plants [8]. India is one of the 12 mega biodiversity zones in the world. The existence of 16 different agro-climatic zones and 10 vegetative regions render its biodiversity unmatched. The existence of two "biodiversity hotspots" makes India rich in biological wealth. More than 45,000 plant species, 15,000–18,000 flowering plants, 23,000 fungi, 2,500 algae, 1,600 lichens, 1,800 bryophytes and 30 million micro-organisms exist in India [94]. Out of 17,000 species, about 7,500 species of higher plants are renowned for their medicinal value, and surveys have suggested that among 45,000 different plant species 15,000–20,000 plants have good medicinal value. Table

2.1 shows the list of some commercially important medicinal plants grown in India (adapted from Ved and Goraya, 2007).

Table 2.1 List of some commercially important medicinal plants grown in India [95]

Common name in Hindi	Latin name	Plant family
Chirata	*Swertia chirata* Buch.Ham.	Gentianaceae
Kalmegh	*Andrographis paniculata* (Burm.f.) Wall. ex Nees	Acanthaceae
Safed musali	*Chlorophytum borivilianum* Sant. et Fernand.	Liliaceae
Ashok	*Saraca indica* L.	Leguminosae
Daru haldi	*Berberis aristata* DC	Berberidaceae
Kokum	*Garcinia indica* Choisy	Clusiaceae
Sarpagandha	*Rauvolfia serpentina* (L.) Benth. ex Kurz	Apocynaceae
Ashwagandha	*Withania somnifera* L.	Solanaceae
Giloe	*Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms	Menispermaceae
Kuth	*Saussurea lappa* (Decne.) C.B. Clarke	Asteraceae
Atees	*Aconitum heterophyllum* Wall	Ranunculaceae
Gudmar kutki	*Gymnema sylvester* R. Br.	Asclepiadaceae
Shatavari	*Asparagus racemosus* Wild	Asparagaceae
Bael	*Aegle marmelos* (L.) Corr.Serr.	Rutaceae
Guggul	*Commiphora mukul* (Stocks) Hook.	Burseraceae
Makoy	*Solanum nigrum* L.	Solanaceae
Tulsi	*Ocimum sanctum* L.	Lamiaceae
Bhumi amla	*Phyllanthus niruri* L.	Phyllanthaceae

Mulethi	*Glycyrrhiza glabra* L.	Fabaceae
Vai vidang	*Embelia robusta* Roxb.	Myrsinaceae
Brahmi	*Bacopa monnieri* L. Pennell	Scrophulariaceae
Jatamansi	*Nardostachys grandiflor* DC	Valerianaceae
Patharchur	*Coleus barbatus* B	Laminaceae
Vatsnabh	*Aconitum heterophyllum* Wall	Ranunculaceae
Chandan	*Santalum album* L.	Santalaceae
Kalihari	*Gloriosa superba* L.	Liliaceae
Pippal	*Ficus religiosa* L.	Moraceae

The alternative health systems like Ayurveda, Yoga, Unani, Siddha, Homeopathy, and Naturopathy play a crucial role in the Indian healthcare system where around 70 % of the population relies on plants or their products for their health care needs [102].

In 1985, Farnsworth et al. identified 117 natural substances from higher plants that were being used globally as drugs [99, 103]. Some of these natural substances are listed in Table 2.2.

Table 2.2 Examples of plant drugs obtained from higher plants

Active Ingredient	Source Plant	Medicinal property
Quinidine	*Cinchona ledgeriana*	Suppresser of out-of-sequence heartbeats
Quinine	*Cinchona ledgeriana*	Antimalarial
Pilocarpine	*Pilocarpus jaborandi*	Glaucoma treatment
Picrotoxin	*Anamirta* sp.	Nervous system stimulant
L-Dopa	*Mucuna* sp.	Anti-Parkinson's disease
Bromelain	*Ananas comosus*	Anti-inflammatory
Scopolamine	*Datura metel*	Sedative
Digoxin	*Digitalis lanata*	Cardiotonic
Atropine	*Atropa belladonna*	Powerful pupil dilator
Curare	*Chondrodendron* sp.	Muscle relaxant

Ephedrine	*Ephedra sinica*	Decongestant
Sennosides	*Senna* sp.	Laxative
Caffeine	*Camellia sinensis*	Central nervous system stimulant
Cocaine	*Erythroxylum coca*	Local anaesthetic
Colchicine	*Colchium autumnale*	Antigout
Podophyllotoxin	*Podophyllum peltatum*	Topical treatment for condylomata acuminata
Reserpine	*Rauwolfia serpentina*	Antihypertensive
Quabain	*Strophanthus gratus*	cardiotonic
Codeine	*Papaver somniferum*	Analgesic/antitussive
Digitoxin	*Digitalis purpurea*	Cardiotonic

Despite all their medicinal value, most of medicinal plants are still subjugated with little or no respect to the future. As noted, intensifying consumer mandate is now subsequent in the undiscerning harvest of wild plants. This is detrimental to both ecosystems and their prized biodiversity.

The apprehension is escalating that countless medicinal plants are on the edge of annihilation. The inevitability to shield these rare medicinal plants seems to be vital. Samples collected today may in the future be found to battle feared diseases, but then again there is no assurance that the plant will then still exist.

Even though not ample is being done to shelter medicinal plants, a few governments are trying to protect some local species. Their labours embrace refining the methods of collection as well as the deliberate cultivation of the plants. The goal is typically to certify appropriate quality control and to regulate commerce for the protection of both manufacturer and customer. These few governments are also involved in educating their inhabitants and in generating larger consciousness of the importance of medicinal plants. For example, in India:

- The Central Council for Research in Ayurvedic, Unani and Siddhi medication and homoeopathy take on Research and Developments into botanicals.
- The Indian Pharmacopoeia Laboratory scrutinizes traditional healing-plant materials.
- The Indian Forestry Service and the Forestry Research Institute both have agendas on the planting and encouragement of medicinal herbs in the forest floor.

- The Botanical Survey of India includes medicinal plants in its assessments of the Subcontinent's plant capitals.
- The State Department of Tribal Welfare sanctions herbal medicines because the tribal peoples incline on these more than most Indians.
- The Arya Vaidya Sala, a vital epicenter of Ayurveda medicine at Kattakkol in Kerala, runs a college, hospital, factory, and research laboratory for medicinal plants. It also runs a herbal garden in addition to two farms that nurture medicinal plants.
- The Tropical Forest Research Institute at Jabalpur in Madhya Pradesh pressures on medicinal plants that can be cultivated amidst the trees. It encourages and distributes plant materials to pharmaceutical enterprises.

Globally little has been done for the conservation of medicinal plants. Despite the increase in trade of medicinal plants, few international and national policies have been devised which encourage cultivation programs or protect the resource base. Furthermore, since the 1988 Chiang Mai Declaration *Saving Lives by Saving Plants* [104], limited genetic protection efforts now embrace species used in traditional medicine. Of course, many countries have "gene banks", but these repositories are mainly devoted to food-crop germplasm and only few contain any medicinal plants [92].

Privately funded international agencies which include the World Conservation Monitoring Center (WCMC), the World Wildlife Fund for Nature (WWF), the Nature Conservancy, the International Union for the Conservation of Nature (IUCN), and several botanic gardens (notably, Kew, Edinburgh, New York, and Missouri Botanical Gardens) seem to be the present leaders in safeguarding medicinal plant biodiversity, or at least in accentuating the need to do so. One remarkable effort is the cooperative alliance between IUCN, WWF and the International Plant Genetics Resource Institute (IPGRI), which has drawn up guidelines for founding a system of wild-species seedbanks in botanic gardens [92].

It seems like improbable that medicinal plants will decline in reputation anytime momentarily. For one thing, the amount of people demanding greater access to herbal preparations in the developed countries seems likely to go on snowballing. In the 21st century, the status of medicinal herbs has increased significantly as large numbers of people seek relatively safe remedies and approaches to healthcare. The mandate for herbal medicines, herbal health products, herbal pharmaceuticals, nutraceuticals, food

supplements and herbal cosmetics etc. is swelling worldwide owing to the mounting appreciation of these products as mainly non-toxic, having scarcer side effects, better compatibility with physiological flora, and availability at affordable prices [105, 106].

2.2 *Picrorhiza kurroa*

P. kurroa known for its hepatoprotective and immuno-modulating properties is an important medicinal herb in the Ayurvedic system of healthcare. The plant belongs to Scrophulariaceae family, one of the largest plant families comprising of 190 genera and 4,000 species. *P. kurroa* is found in the North-western Himalayan region at an elevation of 3,000–5,000 meters [45, 107]. *P. kurroa* plants are perennial, herbaceous and possess an extensive, creeping rootstock and aerial parts comprising of basal leaves and flowering scape only [107]. *P. kurroa* is generally found growing in rock cervices and soggy, sandy soil. *P. kurroa* plants begin to appear with melting of the snow during summer and have a life cycle of 4–5 months.

2.3 Botanical classification and morphology of *P. kurroa*

Division: Magnoliophyta

Class: Magnoliopsida

Order: Solanaceae

Family: Scrophulariaceae

Genus: *P.*

Species: *kurroa*

Figure 2.1 Mature *P. kurroa* plant

P. kurroa propagates vegetatively through tolons, which initially emerge as a young bud, grows into a mature stolon and eventually into rhizome with independent shoots and roots. Distinctly appearing *P. kurroa* plants are actually joined together by stolons beneath the surface [107]. The detachment of stolons from the mother stock results in a completely independent plant. The simple leaves arising from the tip of the upturned stolons are present in rosettes or whorls. Cauline leaves are present only during the flowering phase. The inflorescence is indeterminate terminal spike forming a more or less triangular head. The flowers are sessile, zygomorphic, bilipped, bisexual and purple borne in the axils of small green colored bracts opening during June to September. Sepals persist up to capsule development stage and are of unequal in size [107].

Filaments, generally colorless during the bud stage, turn purplish after anthesis to attract insects for pollination. The flower structure mostly favors cross-pollination. Although exerted stamens are a step towards cross-pollination, introrse anthers ensure that some pollen may fall on the flowering spike itself so as to affect self-pollination [107]. But we know that *P. kurroa* occurs at high altitudes with very short growing seasons [108]. In environments, as in high alpine or arctic regions and deserts, those are poor in because of adverse conditions; autogamy is often the only possible way of sexual reproduction [109, 110]. However, in *P. kurroa*, the exerted stamens, as well as introrse anthers, ensure both allogamy and autogamy.

2.4 History of *P. kurroa*

Picrorhiza is a small genus belonging to family Scrophulariaceae and the tribe Veroniceae. Scrophulariaceae family is placed in the order Scrophulariales, subclass Asteridae and Dicotyledonae class of Angiosperm, according to the taxonomical system of Cronquist. The genus *P.* was supposed to be monotypic, with *P. kurroa* as the only species, until a second species, *P. scrophulariiflora* (originally written as "*P. scrophulariaeflora*") was reported [111]. Later, this new species was brought under a separate genus and renamed to *Neo P. scrophulariiflora* [112]. This name was accepted as the official name for this species [113].

P. kurroa was reported for the first time by Royle on August 24^{th,} 1835 (Figure 2.2) in "Illustrations of Botany" [114]. Later, Bentham on November 17^{th,} 1835 described the genus and the species in his book "Scrophularineae Indicae" [115]. Since Bentham

was the first to describe the species, the accepted species name has been "*P. kurroa* Bentham", or "*P. kurroa* Royle ex Bentham".

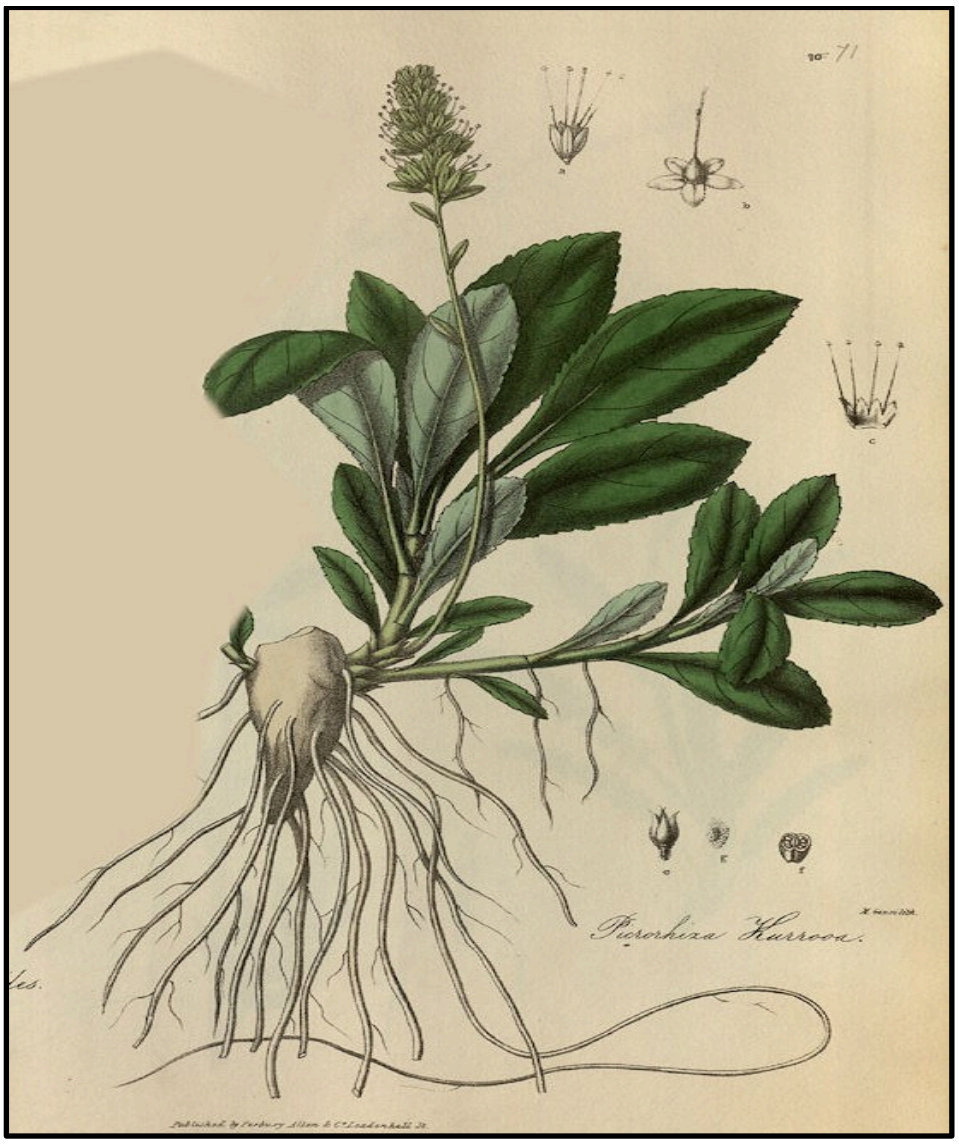


Figure 2.2 Photograph showing drawing of *P. kurroa* published by Royle in 1835 [114]

2.5 Geographic distribution of *P. kurroa*

P. kurrooa cultivates naturally in various places from Kashmir to Kumaon [111] and from Pakistan to Uttarakhand [116]between an altitudinal range of 3000 – 5000 m amsl (Figure 2.3 and Table 2.1)

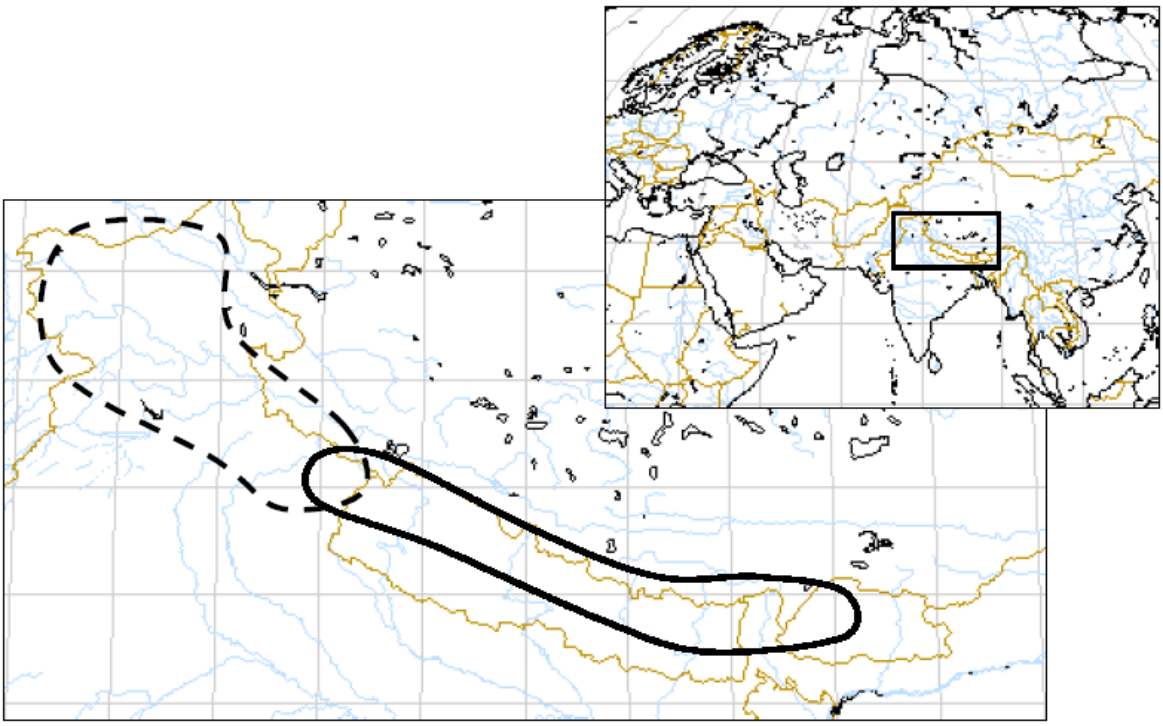


Figure 2.3 Distribution of *P. kurroa Royle* (...) and *P. Scrophulariiflora Pennell* (--) in Himalayan region [117].

Table 2.3 Distribution of *P. kurroa* in India. State and places in respective states have been given in separate columns

State	Location	Reference
Jammu and Kashmir	Apharwat, Kashmir	[118]
	Burzil Pass	[111]
	Gumri	[119]
	Kamri Pass (Naigund)	[111]
	Kolohi; Zojpal; Sonsa Nag	[120]
	Lipper Valley, northwest of Kashmir Valley	[121]
	Mir Panzil Pass, Deosai Road,	[111]
	Nafran	[111]
	Pahlgam	[111]
	Pir Panjal range; Krishan Ganga valley; upper Lidder valley	[122, 123]
	Simthan, Jammu	[124]

	Sonamarg	[111]
	Tragbal	[111]
	Zojibal Pass	[111]
Himachal Pradesh	Basharh: Chhit Kul; Hangla Pass; Pieri Pass, Kinnawar	[111]
	Chafuwa, Talla Johar, Eastern Kumaon	[125]
	Chamba and Kangra	[126]
	Chamba: Kukti Pass	[125]
	Chamba; Kinnaur; Kulu; Lahul; Spiti	[127]
	Chhakinal watershed, District Kullu	[128]
	Kangra (Kulu): Chandrokani; Sumdo	[111]
	Lahaul; Kinnaur; Kulu; Rohru; Kangra; Pangi-Bharmour	[129]
	Lahul: Dilburig, Chenab Valley; Kyelang	[111]
	Manimahesh; Rotang Pass; Lahaul Spiti Forest Division	[130]
	Pangi valley; Birmor valley; Lahaul valley; Dauladhar valley	[122]
	Pangi; Bharmour; Lahaul; Kalpa; Dauladhar; Barabhangal ranges	[123]
	Rohtang slope, Lahaul-Spiti	[131]
	Rudranath Bugyal, Chamoli, Garhwal	[132]
	Satrundi, Chamba	[118]
Uttar Pradesh/ Uttarakhand	Around Agora-Dodital, Uttarkashi district	[133]
	Bhillangna Valley, Sahasru Tal, Tehri-Garhwal	[134]
	Buhna, Nar Parbat, Garhwal	[118]
	Damdar Valley, Tehri-Garhwal	[134]
	Dayara, Devkund	[135]
	Deodi Ramani, Garhwal,	[134]
	Enroute Rupkund, Garhwal,	[134]

	Gangotri, Tehri-Garhwal	[134]
	Harsil, Raithal, Sukhi, Sayara, Tehri-Garhwal	[134]
	Jumnotri, Tehri-Garhwal	[118]
	on the way to Yumnotri, Tehri-Garhwal	[134]
	Kedar Konta, east of Sutlej gorge	[136]
	Kedar Nath; Herker Dun; Ponwali; Tali; Harsil, Garhwal Himalaya	[137]
	Kedarkanta, Tehri-Garhwal	[134]
	Kedarnath, Har-ki-dun, Ponwati, Tali, Harshil and Gangotri in Garhwal Hills	[123]

2.6 Vernacular names

In Chinese system of medicine, *P. kurroa* is known as 'Hu Huang Lian'. In India *P. kurroa* is known by different commercial and regional names; it is called ‘Tikta’, ‘Tiktarohini’, ‘Kavi’, ‘Sutiktaka’, ‘Kauka’ and ‘Rohini’ in Sanskrit, ‘Katki’ and ‘Kutki’ in Assamese, ‘Hellebore’ in English, ‘Kadu’ and ‘Katu’ in Gujarati, ‘Kutki’ in Hindi, ‘Katuka rohini’ in Kannada, ‘Kaduk rohini’ in Malayalam, ‘Kalikutki’ in Marathi, ‘Katuki’ in Oriya, ‘Karru’ in Punjabi, ‘Kadugurohini’ in Tamil, ‘Karukarohini’ in Telugu and ‘Kutki’ in Urdu. It is widely known as 'Kour' in Kashmir Himalaya, 'Kadu' in Himachal Himalaya and 'Kadvi' in Uttrakhand Himalaya.

2.7 Medicinal properties of *P. kurroa*

Extracts obtained from *P. kurroa* have been known to possess a great variety of medicinal applications some of which are listed below

2.7.1 Hepatoprotective

The hepatoprotective effect of *P. kurroa* extract is chiefly due to an increase in the activities of antioxidant enzymes which are responsible for free radical scavenging. A study demonstrated that aqueous extract of *P. kurroa* with high antioxidant activity, as confirmed using different radical scavenging assays, was effective in subduing the deleterious effects of ethanol. Adding of *P. kurroa* aqueous extract along with ethanol restored the activities of antioxidant enzymes and significantly reduced lipid peroxidation [138].

2.7.2 Anti-tumor/Anti-neoplastic activity

Anti-tumour and anti-carcinogenic activity of *P. kurroa* extract were evaluated in mice. The extract reduced the volume of transplanted solid tumours induced by Dalton's lymphoma ascites (DLA) tumour cell lines and increased the lifespan of an ascites tumour bearing mice. *P. kurroa* extract inhibited yeast topoisomerase I and II enzyme activity when tested on *Saccharomyces cerevisiae* mutant cell cultures [139]. In another study, anti-neoplastic activities of methanolic and aqueous extracts of *P. kurroa* rhizome were investigated. The cytotoxicity of the extracts was tested by XTT assay in MDA-MB-435S (human breast carcinoma), Hep3B (human hepatocellular carcinoma) and PC-3 (human prostate cancer) cell lines. The extracts were cytotoxic at the tested dosage and were able to target cells towards apoptosis [140].

2.7.3 Antioxidant activity

Antioxidant effects of *P. kurroa* were studied in rats treated with a tumour treating drug, Adriamycin, known to cause severe cardiomyopathy. Oral administration of *P. kurroa* extract (50 mg/kg body weight/day, for a period of 15 days) significantly prevented all adriamycin-induced adverse effects and maintained the rats at normal status. This protective effect of *P. kurroa* might be attributed to its membrane-stabilizing property and/or antioxidant nature [141].

2.7.4 Immuno-modulatory/Adjuvant activity

RLJ-NE-205, a biopolymeric fraction isolated from the rhizomes of *P. kurroa*, was evaluated for its effect on the in vivo immune function of a mouse with sheep red blood cells as an antigen. At a quantity of 50 mg/kg, a significant increase in lymphocyte proliferation and cytokine levels was experiential signifying that the fraction RLJ-NE-205 amended the immune response and could be regarded as a biological response modifier [142].

2.7.5 Antiasthmatic activity

In a study by Dorsch et al., 1991, phenol glycoside androsin derived from *P. kurroa* was identified as an active compound which prevented allergen and platelet-activating factor-induced bronchial obstruction in guinea pigs [55]. The ethanolic extract of the roots of *P. kurroa* was evaluated for antiasthmatic activity both *in-vivo* and *in-vitro* in guinea pigs. Animal studies used the histamine-induced broncho-constriction in guinea pigs. The

effect of the ethanolic extract of the plant on isolated guinea pig ileum showed the extract is effective against histamine and acetylcholine-induced contraction. The results revealed that the ethanolic extract produced antiasthmatic activity due to the presence of saponins and flavonoids [143].

2.7.6 Viral Hepatitis

In a study, D-galactosamine was used to induce hepatitis in rats, an animal model of both drug-induced hepatitis and viral hepatitis of human beings. Administration of an alcoholic extract of *P. kurroa* prior to D-galactosamine induced hepatitis significantly prevented the D-galactosamine-induced decreases in the levels of protein and glycoprotein and in the activities of superoxide dismutase and catalase. The antihepatotoxic potential of *P. kurroa* might be due to the antioxidant and hypolipidemic nature of *P. kurroa* [144]. In another study, *P. kurroa,* anti-hepatitis B-like activity was investigated [145]. *P. kurroa* was found to possess a promising anti-hepatitis B surface antigen activity. Levels of bilirubin, SGOT and SGPT were significantly lower in the treatment group, and the time required for bilirubin values to drop to 2.5 mg was 27.4 days in the treatment group versus 75.9 days for the placebo group.

2.7.7 Anti-inflammatory activity

In a study by Singh et al., 2006, powdered root of *P. kurroa,* its alcoholic extract along with active constituents kutkin, picroside-I and kutkoside were demonstrated to have an anti-inflammatory activity [146]. Substantial anti-inflammatory activity was detected in adjuvant-induced arthritis in rats and mice. In carrageenan-induced oedema in rats and mice, inhibitory activity was remarkably heightened with intraperitoneal treatment. Kutkin exhibited significant action in dextran-induced oedema in rats. It inhibited acetic acid induced vascular permeability in mice and leucocyte migration in rats.

2.7.8 Anti-allergic and Anti-anaphylactic

25mg/kg dose of Picroliv, a standardized iridoid glycoside fraction obtained from the root and rhizome of *P. kurroa,* was found to inhibit passive anaphylaxis in mice (82%) and rats (50–85%) and also safeguarded mast cells degranulation (60–80%) in a concentration dependent manner [147].

2.7.9 Anti-cholestatic activity

Picroliv, administered orally for 21 days at a dose level of 25/mg/kg/day to cholestatic rats substantially prevented the biochemical changes induced in liver and serum of cholestatic rats suggesting that anti-cholestatic activity may be ascribed to antioxidant property or it's specific role in protein synthesis [148].

2.7.10 Potential drug against NAFLD

Metabolic insults to liver arising due to diseases, toxins, obesity, type 2 diabetes mellitus result in the development of non-alcoholic fatty liver disease (NAFLD). A new NAFLD model was developed in male Wistar rats by giving them a high-fat diet for 2 weeks to induce NAFLD. *P. kurroa* extracts brought about a reversal of the fatty infiltration of the liver and also lowered the quantity of hepatic lipids in comparison to the control group indicating its potential as a treatment drug for NAFLD [149]. Table 2.4 summarizes the medicinal properties of *P. kurroa*.

Table 2.4 Medicinal value of *P. kurroa*

Medicinal Properties	**References**
Hepatoprotective	[138]
Anti-tumor/Anti-neoplastic activity	[56, 140]
Antioxidant activity	[141]
Immuno-modulatory/Adjuvant activity	[142]
Antiasthmatic activity	[55, 143]
Viral Hepatitis	[144, 145]
Anti-inflammatory activity	[146]
Anti-allergic and Anti-anaphylactic	[147]
Anti-cholestatic activity	[148]
Potential drug against NAFLD	[149]

2.8 Medicinal properties of pure chemical constituents of P. *kurroa*

2.8.1 Picroside-I

Very few reports deal with the pharmacological properties of picroside-I. Column chromatography purified picroside-I was used to demonstrate anti-invasion activity against MCF-7 cell lines. It exhibited considerable cytotoxic potential in a dose-dependent manner establishing it as a valuable anti-invasive drug candidate for cancer therapy by suppressing Collagenases and Gelatinases. Suppression of the inflammatory mediators was also demonstrated [150]. In another study by Zhou role of picroside-I in the effective treatment of hepatitis B was established [151].

2.8.2 Picroside-II

In contrast to picroside-I, several reports exist related to the pharmacological properties of picroside-II. A study reported that intravenous treatment with picroside II might be beneficial to inhibit neuronal apoptosis and hence improve the neurological function of rats upon cerebral ischemia-reperfusion injury [152]. A similar study by Pei et al. also reported the treatment of cerebral ischemic injury in rats by injecting picroside-II intraperitoneally [153]. This effect might be due to the fact that picroside-II could down-regulate the expressions of TLR4, NFκB and TNFα to inhibit apoptosis and inflammation induced by cerebral ischemic reperfusion injury and thus improving the neurobehavioral function of rats [154]. Picroside-II has also been reported to protect hepatocytes against injury and from apoptosis where it might be participating in the up-regulation of the bcl-2 gene expression and antioxidation [155]. Picroside-II has likewise been shown to relieve hepatocytes from injuries induced by CCl4 (carbon tetrachloride), D-GalN (D-galactosamine) and AP (acetaminophen), by scavenging free radicals, protecting normal constructions of mitochondrial membranes and enhancing the activity of ATPase in mitochondria, thereby modulating the balance of liver energy metabolism [156]. In addition to picroside-II role in reducing apoptosis in neuronal cells and hepatocytes, cardioprotective role of picroside-II has also been explored wherein picroside II evidently lessened hypoxia/reoxygenation-induced cell damage, which was apparent by the augmented cell viability and the equivalent reduction in lactate dehydrogenase release (LD) [157]. The medicinal properties of the two chief chemical constituents picroside-I and II have been compiled in Table 2.5.

2.8.3 Apocyanin

Apocyanin (4-hydroxy-3-methoxy-acetophenone), another constituent of the Himalayan herb *P. kurroa* is an acetophenone to which a range of biological activities have been attributed [158]. It is a potent anti-inflammatory compound. Apocyanin is an inhibitor of the intracellular translocation of two critical cytosolic components of the NADPH-oxidase complex present in the cell membrane [159]. In this regard, the effectiveness of apocyanin in the treatment of respiratory diseases may have been derived from its ability to inhibit peroxynitrite ($ONOO^-$) formation [160], which has been proposed to induce epithelial damage, mediator release and accordingly hyperresponsiveness [161]. Apocyanin has been found to be useful in the treatment of inflammatory diseases induced by oxidative stress through NOX activity [162].

2.8.4 Catalpol

Catalpol, an iridoid glucoside has been reported to induce neuronal differentiation in PC12h cells through activation of the intracellular signal transduction pathway [163], conferring it a neuroprotective role. Treatment of PC12 cells with catalpol can block H_2O_2-induced apoptosis by the regulation of Bcl-2 family members, as well as suppression of cytochrome c release and caspase cascade activation [164]. Catalpol has also been reported to exert neuroprotection effect on gerbils of transient global ischemia [165], attenuate apoptosis, rescue hippocampal CA1 neurons and reduce cognitive impairment [166]. A study showed that the neuroprotective effects of catalpol on the mice brain tissue increased the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), decreased the malondialdehyde (MDA) level, raised the actions of Na+–K+ ATPase and Ca^{2+}–Mg^{2+} ATPase on the cerebral cortex and hippocampus of D-galactose treated mouse [167] .

Table 2.5 Medicinal Importance of P-I and P-II

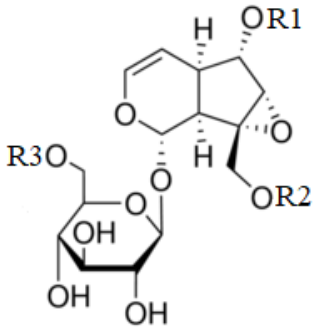
Chemical Constituents	**Pharmacological Properties**
Picroside-I	Anti-microbial [150] Used against hepatitis B [168]

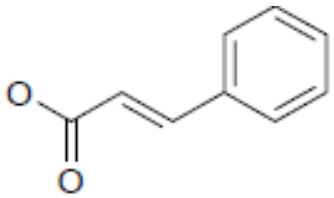
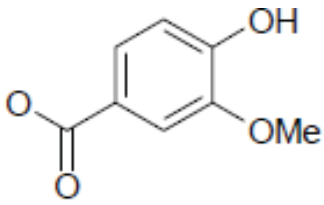
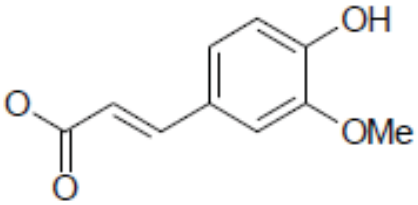
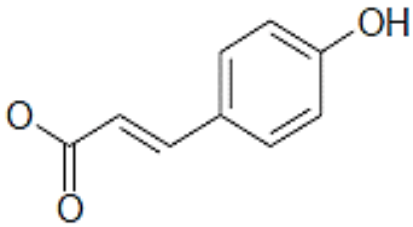
Picroside-II	Protects cardiomyocytes against oxidative-stress injury induced by Hypoxia [153] Inhibits neuronal apoptosis [152] Anti-inflammatory activity [154]Prvents hepatocytes from apoptosis [155] Reduce NO (Nitric oxide) concentration and enhance GSHPx activity [169] Prevents cerebral ischemic reperfusion injury [170] Prevents myocardial ischemia-reperfusion injury [171]

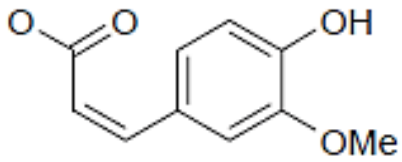
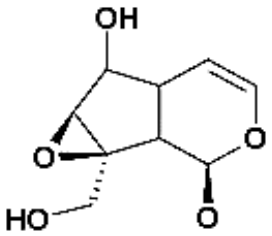
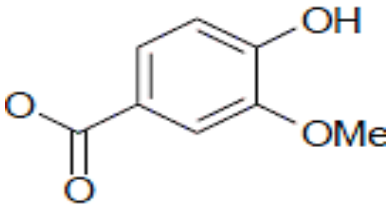
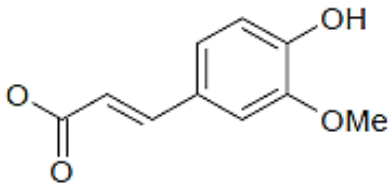
2.9 Phytochemistry of *P. kurroa* − An ocean of phytochemical constituents

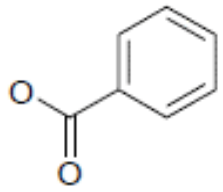
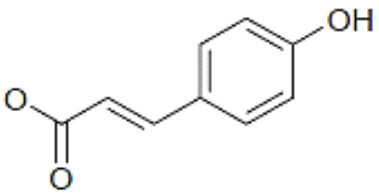
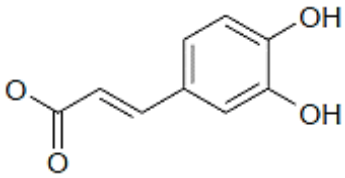
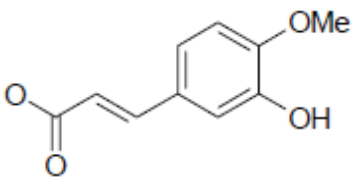
P. kurroa accrues innumerable types of secondary metabolites such as iridoid glycosides, cucurbitacins, secoiridoids, and phenolics. Amongst these constituents, picrosides, belonging to the iridoid class of secondary metabolites are the chief constituents. While iridoids and secoiridoids represent a large group of monoterpenes, cucurbitacins are triterpenoid in nature. In addition to the picrosides P-I and P-II, *P. kurroa* synthesizes additional iridoid compounds such as picroside-III, picroside-IV, picroside-V, catalpol, kutkoside, 6-feruloylcatalpol, Veronicoside, specioside, verminoside, minecoside, aucubin and pikuroside.

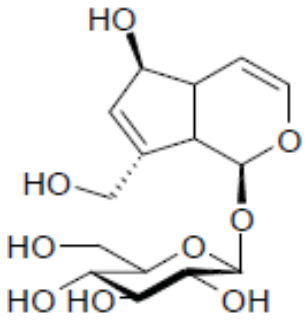
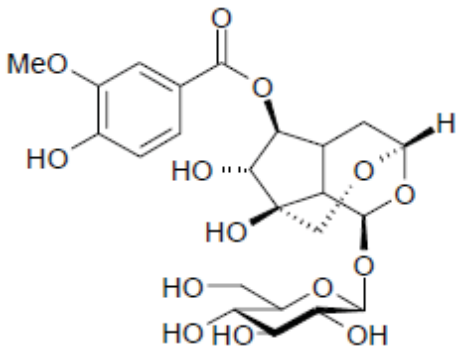
Table 2.6 Iridoid glycosides isolated from *P. kurroa*

 **Basic Structure of picrosides**	**[52]**

R1 = H, R2 = H, R3 =  **Picroside-I**	**[172]**
R2 = H, R3 = H, R1 =  **Picroside-II**	**[173]**
R1 = H, R2 = H, R3 =  **Picroside-III**	**[174]**
R1 = H, R2 = H, R3 =  **Picroside-IV**	**[175]**

R1 = H, R2 = H, R3 =  **Picroside - V**	**[176]**
 **Catalpol**	**[173]**
R1 = H, R3 = H, R2 =  **Kutkoside**	**[65]**
R2 = H, R3 = H, R1 =  **6-feruloylcatalpol**	**[177]**

R2 = H, R3 = H, R1 =  **Veronicoside**	**[177]**
R2 = H, R3 = H, R1 =  **Specioside**	**[175]**
R2 = H, R3 = H, R1 =  **Verminoside**	**[175]**
R2 = H, R3 = H, R1 =  **Minecoside**	**[177]**

 **Aucubin**	**[173]**
 **Pikuroside**	**[178]**

2.9.1 Cucurbitacins

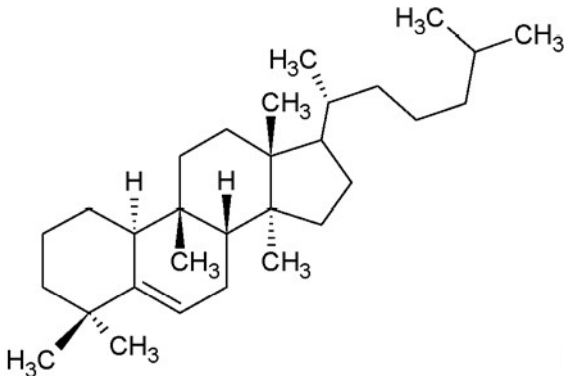


Figure 2.4 The basic skeleton of cucurbitacin

Cucurbitacins are a group of tetracyclic triterpenoids originally discovered in the plant family of *Cucurbitaceae.* Cucurbitacin containing plants are known for their antipyretic, analgesic, anti-inflammatory, antimicrobial, and antitumor activities [179, 180]. Cucurbitacins are classified into 12 main categories on the basis of variations in their side-chain [180]. There are 17 main molecules from cucurbitacin A to cucurbitacin T, along

with hundreds of derivatives obtained from them. Among these, cucurbitacin B, D, E, I, and their derivatives have been studied extensively for their strong anticancer activities [179, 180] along with cucurbitacin F, O, P, Q, and their derivatives which are known to have modest anticancer activities [180]. After being neglected for decades, cucurbitacins are once again attracting attention as a potential anticancer drug. Its role as a JAK/STAT inhibitor, a MAPK modulator, and a cytoskeleton disruptor makes it an excellent candidate for clinical investigation.

2.9.2 Iridoids

Iridoid glycosides possess a basic cyclopentano-[*c*]-pyran skeleton, also known as an iridane skeleton (*cis*-2-oxabicyclo 4, 3, O, nonane). This is formed by 8-*epi*- iridodial's ring closure (Figure 2.5) and usually occurs as glycosides (sugar bound to a non-carbohydrate moiety).

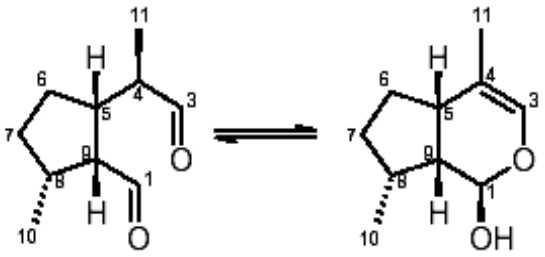


Figure 2.5 Chemical Structure of 8-*epi*-iridodial.

The sugar part of glycoside is called glycone whereas non-sugar part is known as an aglycone. Iridoids generally have nine carbons in the iridoid ring and a tenth carbon is often bonded to either C-4 or C-8 position. This carbon could be a methyl group, carbonyl group or secondary alcohol. A methyl group (C-10) is found most commonly at the C-8 position and is rarely absent (Figure 2.6 A).

The term iridoid was derived from the name iridomyrecin, iridolacton, and iridodial, compounds that are present in the defence secretions of certain ant species of the *Iridomyrmex* genus. Different authors have reviewed their occurrence [181, 182], distribution [183], biosynthesis [184], and biological activities [185, 186].

The iridoids are grouped into four main categories namely aglycone iridoids, secoiridoids, bis-iridoids and iridoid glycosides. Introduction of additional carbons, functional groups, and double bonds into the skeleton creates more diverse and complex iridoid structures.

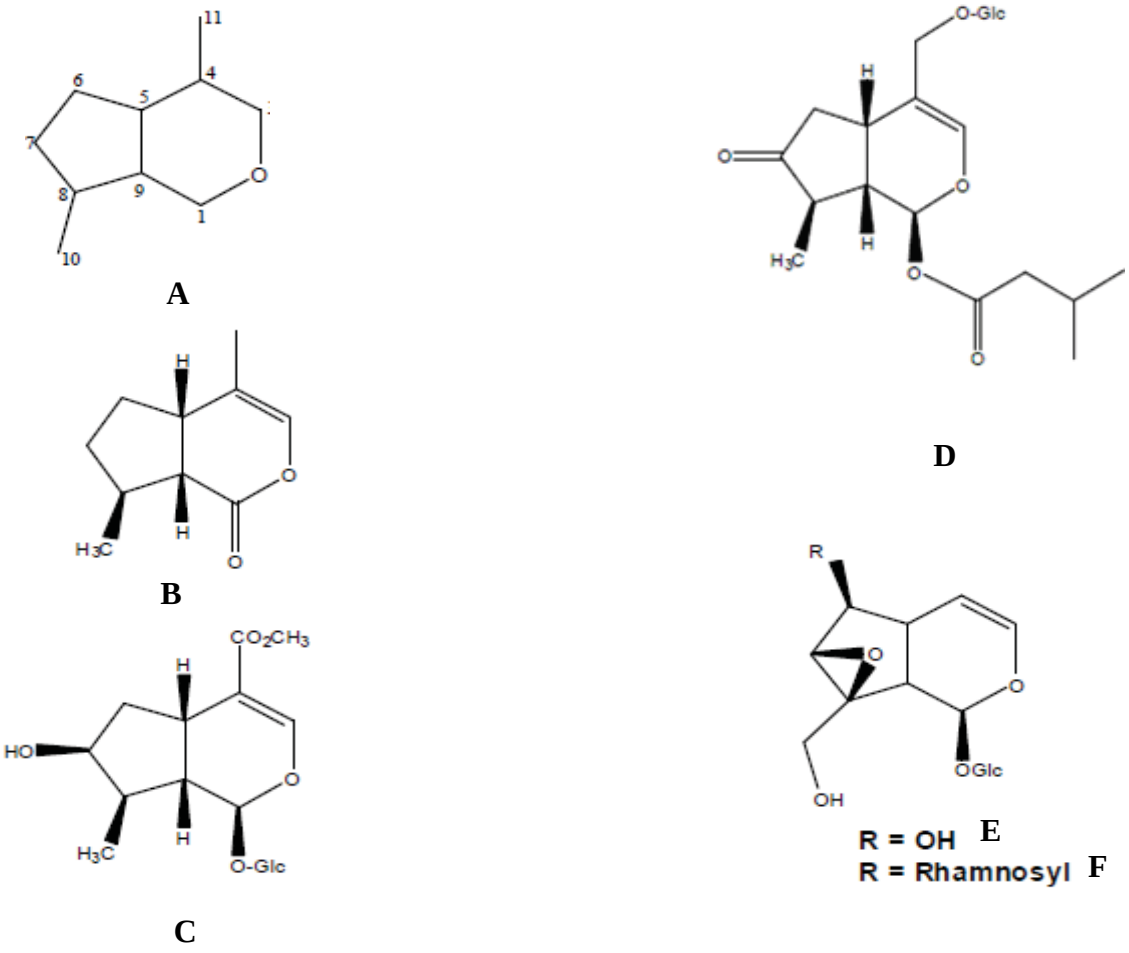


Figure 2.6 Different types of iridoid aglycone and aglycone catalpol found in plants

Aglycone structures like nepetalactone (Figure 2.6 B) was isolated from *Nepeta cataria* (family, Lamiaceae) and is commonly known as catnip, an attractant of cats [187]. However, most non-glycosidic iridoids form part of modified structures such as alkaloids, polycyclic compounds, polyesters and intramolecular ethers [188].

A large number of iridoid glycosides can be characterized as glycosides with a glycosidic association between the anomeric hydroxyl of D-glucose and the C-1 hydroxyl of aglycone. A simple example of this is loganin (Figure 2.6 C) isolated from *Odontites verna* subspecies *Serotina* (family, Scrophulariaceae) which act as a biosynthetic precursor of many iridoids. A glycosidic connection between the C-11 hydroxyl of the aglycone and anomeric carbon hydroxyl of D-glucose is a rare occurrence. This can be noticed in ebuloside (Figure 2.6 D) isolated from *Sambucus ebulus* (family, Caprifoliaceae) [189]. Type of sugar and its complexity varies in different iridoid glycosides, for example, catalpol (Figure 2.6 E) isolated from *Verbascum saccatum*

(family, Scrophulariaceae) has a rhamnosyl attached at C-6 to form 6-*O*- a-L-rhamnosylcatalpol in addition to glucose (Figure 2.6 F).

Secoiridoids is formed by cleavage of 7, 8-bond of the cyclopentane ring (Figure 2.7). Sweroside is a secoiridoid isolated from *Swertia nervosa* (family, Gentianaceae) [190]. Various other structural modifications give rise to different secoiridoids. Aglycone secoiridoids rarely exist in nature [191].

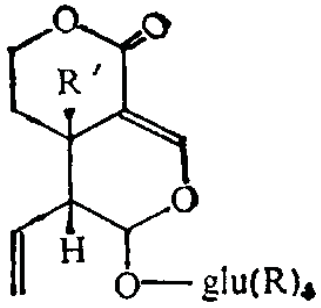


Figure 2.7 Chemical structure of secoiridoids found in plants

Bis-iridoids is formed by dimerization of both iridoids and secoiridoids. Globuloside B (Figure 2.8 A) is a bis-iridoid, isolated from *Globularia meridionalis* (family, Plantaginaceae) [192]. The iridane skeleton and its functional groups also vary in iridoids. The methyl group (C-10) might be oxidized to a secondary alcohol as in catalpol (Figure 2.6 E) or may also undergo epoxidation as in catalpol (Figure 2.6 F).

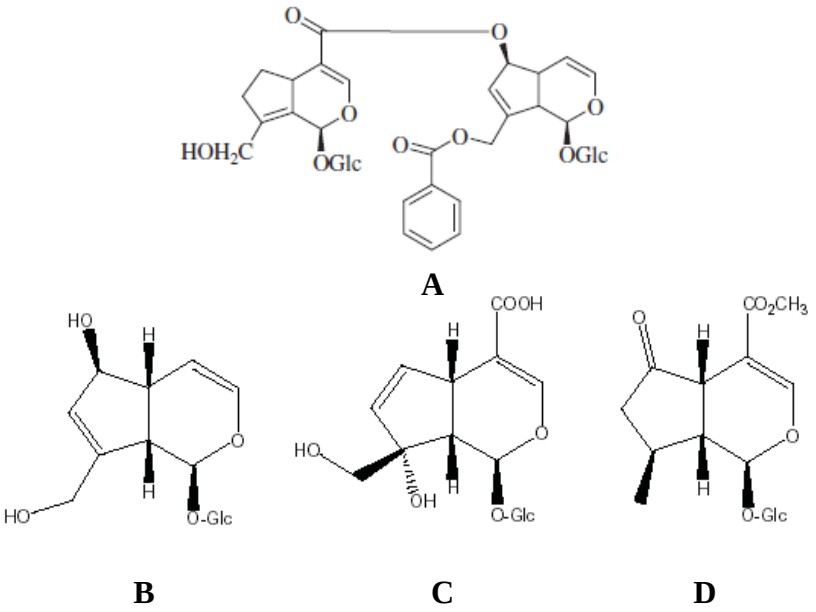


Figure 2.8 Structure of Bis-iridoids found in plants

The presence of double bonds in iridoid ring as in aucubin or monotropein (Figure 2.8 B) isolated from *Tecoma chrysantha* (family, Bignoniaceae) (Figure 2.8 C) also generates more structural diversification of iridoids. The introduction of a hydroxyl at C-6, C-7 and C-8, or oxidation of C-6, C-7 and C-8 as in verbenalin (Figure 2.8 D) isolated from *verbena officinalis* (family, Verbenaceae), is yet another mode of structural diversification.

Iridoids can be carboxylated and non-carboxylated depending upon presence and absence of carboxylate group. These are derived from different biosynthetic precursors [193]. Carboxylated (C-11) are formed from loganin (Figure 2.6 C) and 8-*epi*-loganin (Figure 2.9) [193]. Whereas non-carboxylated iridoids are derived from 8-epi-iridodial (Figure 2.5), 8-epi-iridotrial and 8-epi-deoxyloganic acid; these act as precursors in the formation of decarboxylated carbocyclic iridoids such as aucubin (Figure 2.8 B) and catalpol (Figure 2.6 E).

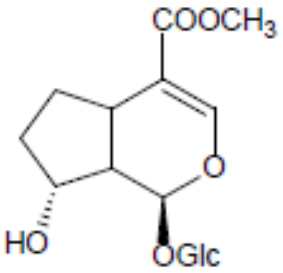


Figure 2.9 Structure of 8- *epi*-loganin

Iridoid glycosides present in *P. kurroa* are collectively termed as picrosides. Several iridoid glycosides have been isolated from *P. kurroa*. Out of which P-I and P-II are major constituents. These are C-9 glycosides and contain epoxy oxide in the ring. These shares similar basic structure except that trans-cinnamyl in P-I and vanilloyl in P-II are at different carbon atoms. Widespread investigation has been devoted to standardized iridoid fractions of *P. kurroa*, *e.g.* kutkin and Picroliv. Kutkin is attained by crystallization of iridoid fraction and entails P-I and kutkoside in a ratio of 1: 2 [65, 194]. Picroliv has similar constituents, but is a less cleansed portion and comprises around 60% of an equal concoction of P-I and kutkoside [66]. More recent publications designate Picroliv as a consistent iridoid fraction containing 60% of P-I and kutkoside in a 1: 1.5 ratio [67]. In all these studies kutkoside was treated as single compound [195], and some studies also mentioned it as P-II [145]. However, Bhandari *et al.*, (2009) stated kutkoside to be a mixture of iridoid glycosides namely; P-II, P-IV and 6-feruloyl catalpol [196].

2.10 Factors affecting the biosynthesis and accumulation of chemical constituents in *P. kurroa*

Innumerable factors such as tissue age, different growth/developmental stages, and environmental variations influence the levels of major chemical constituents being accumulated in *P. kurroa*

2.10.1 Variations in the P-I and P-II levels in relation to tissue age in P. kurroa

An important factor governing the enhanced yield of active principles and biomass production is the age of the plant. Levels of secondary metabolites within a plant fluctuate with age, and there are various reports, which shows age as a significant factor with respect to secondary metabolite content. It has been reported that newer leaves contained higher camptothecin (CPT) concentrations than older leaves. Within a branch, there was a direct waning of CPT concentration from leaves at the apex of the branch down to Leaf 7. Relating leaves of comparable age, those from newer trees had higher CPT concentrations than hose from grownup trees [197]. In a similar report in *Lantana camara,* younger leaves were found to be most vigorous in biosynthesis and accretion of secondary metabolites than the aged leaves. In inference, leaf ontogeny affects the biosynthesis and accumulation of secondary metabolites and in that way their organic properties [198]. In *Olea europea* and *Tamarex aphylla,* the younger plant parts contained enhanced levels of alkaloids in comparison to older parts [199, 200]. Whereas for saponins, aged stems produced greater amounts of saponin as compared to their younger counterparts. An elevated level of saponin is obtained in aged stems of O. *eurpea,* O. *ferruginea,* and *P. glandulos* [201, 202]. Chan *et al.,* [2008] in their study on cotton *(Gossypium hirsutum*) reported that young plant parts generally have a higher level of plant secondary metabolites than mature plant parts. They observed that young expanding foliage had greater terpenoid aldehyde levels in comparison to mature foliage [203].

In case of *P. kurroa,* it has been reported that picrosides were present in the leaf tissues at all the node positions, where the top was designated as the youngest leaf and the bottom taken as a mature leaf. HPLC results revealed that maximum picrosides contents were present in the youngest leaf [204].

2.10.2 Effects of different growth/developmental stages of P. kurroa on P-I and P-II content

Plants are complex organisms involving an extensive diversity of organs, tissues, and cell types. Respectively, these different units are branded by a exclusive and precise progressive programme which is also replicated in their metabolic composition. Owing to procedural limitations, metabolic studies have thus far essentially fixated on intricate plant parts such as shoots and roots, ignoring the alterations in tissues and cell types inside these plant organs. A number of studies have conveyed on associations amongst plant development and the metabolic status of diverse plant organs, such as leaves, roots, flowers, seeds, and fruits [205]. In reasonably fewer studies, different organs have also been compared with each other [206-212]. These studies typify that plants are gifted to synthesize massive diverse secondary metabolites, of which some are more universal and amassing in many tissues whereas others are very explicit for discrete tissues. The exact accrual of metabolites proposes dedicated functions in unlike phases of a plant's growth [213]. Secondary metabolites, have an ample chemical assortment and choice of functions and are often very explicit to certain plant tissues. For example, benzoyloxylated glucosinolates and proanthocyanidins are found solely in seeds, while certain terpenes are specific for flower tissues [214-216]. The cost-effective gathering of metabolites in a tissue-dependent manner permits the plant to devote treasured resources frugally in growth and development and as such contributes to amplified appropriateness and modest capacity [212, 217]. Apparently, the developmental stage of plants and the state of their issues are imitated in the metabolic signature of its compartments. Also, the metabolic composition does not always hinge on *de novo* biosynthesis and catabolism but can also result from a rearrangement of compounds. New leaves, for example, have a very different glucosinolate profile compared to senescent, older leaves, probably due to re-allocation to safeguard the defense of the inner rosette from feeding by herbivores [212]. On a unfathomable level, metabolic alterations can also be pragmatic between explicit cell types, and even organelles, in secluded tissues and organs [218, 219]. Previous findings also disclosed that numerous secondary metabolites were largely restricted in the vacuole (storage) and cytosol (synthesis), while primary metabolites, critical for biochemical pathway directive, were spotted in all compartments [220]. Overall, three-dimensional regulation of metabolism appears to have a prodigious

impact on the metabolic silhouette of varied plant species. Furthermore, even within entities, the metabolic composition reflects the developmental stage and function of the plant's parts. When relating a plant's metabolic status, care should be taken with reverence to unlike organs and cell types within the studied species.

There are very few reports showing the influence of growth/developmental stages of *P. kurroa* on P-I and P-II content and the spatiotemporal differential accumulation of picrosides. Sood & Chauhan [2010] reported that P- I and P-II differential accumulates in shoots and roots of the *P. kurroa* [53]. They also specified that leaves are a respectable source of picrosides and can be used as a reserve for P-I, henceforth, uprooting the whole plant was needless, and accrual of P- I is developmentally controlled in diverse morphologic stages of *P. kurroa.* It has been reported that picrosides were present in the leaf tissues at all the node positions, with the extreme level in the youngest leaf. Nevertheless, it was observed that leaf tissues predominantly contained P-I, also a reasonable analysis of picrosides in different tissues revealed significantly higher P-I content in the leaf tissues as compared to the rhizomes and roots. Roots exhibited a higher amount of P-II as compared to P-I, though the contents of both P-I and P-II were less as compared to that in the leaf. In rhizomes, both picrosides, P- I and P-II were present in analogous amounts [204, 221]. However, Katoch *et al.* [2011] stated that though leaves of *P. kurroa* contain picroside but their concentration is inferior than the picroside of rhizome and root and this buildup is reliant on elevation [222].

2.10.3 Effect of environmental factors on P-I and P-II levels in P. kurroa

Environmental factors such as light intensity, temperature, water availability, type and composition of soil and several other have a substantial influence on the quality and productivity of medicinal plants [223]. Plants of the same species occurring in different environments may differ significantly in their content of particular secondary metabolites [224]. The accumulation of plant secondary metabolites is stalwartly affected by environmental disparity in light, nutrients, temperature, and biotic and abiotic stresses and has been expansively reviewed in recent years [225-231].

2.10.3.1 Effect of light and temperature on picrosides content

It has been reported that *P. kurroa* plants maintained at l5°C show elevated picrosides level as compared to *P. kurroa* plants at 25 ± 1 °C [53]. Bestowing to a report, at l5°C the expression of *pkhmgr* and *pkdxs* was heightened by 216% and 286% respectively, in comparison to those at 25 ± 1 °C [232]. Also, the level of total picrosides was found to be augmented by 22% at 15 ± 1 °C in contrast to the plants maintained at 25 ± 1 °C [232]. Subsequently, the expression of *pkhmgr* and *pkdxs* was observed to be very poor at 25 ± 1 °C, experiments pertaining to the effect of the dark was achieved only at l5°C condition. In an experiment spanned over 12 h, the expression of *pkhmgr* and *pkdxs* was detected to be raised by 70% and 112% in the light as compared to that observed under dark. The level of picrosides in light was enhanced by 163% of what was observed under dark conditions [232]. Sood & Chauhan [2010] also informed ~17 times higher content of picroside I at 15 ± 1 °C vis-à-vis at 25 ± 1 °C as determined by HPLC [53]. Further, to discover whether the picrosides accumulation fluctuates in response to varying light intensities, the level of picrosides was assessed at four different time points of the day. The level of picrosides was higher during light period (at 9:00 h and 13:00 h) as compared to the low light/dark period [204].

2.10.3.2 Effect of altitude on picrosides content

The erraticism of chief phytoconstituents within the same species at different altitudinal ranges indicate a significant relationship between the quality and quantity of active principle and the environmental factors such as changed habitat and trauma conditions of different geographical locations. A broad range of environmental factors changes with the elevation of the natural growing site. These factors include precipitation, mean temperature, daily thermal amplitudes, soil fecundity, wind speed, temperature extremes, atmospheric pressure, duration of snow-cover, length of the vegetation period, and radiation intensities. Numerous abiotic and biotic factors change with the altitude of the growing site. Abiotic factors are mainly climatic (decreasing temperatures, increasing radiation under clear sky conditions, increase of cloud cover in mid-altitudes, increasing wind speed in high altitudes) but also include soil characteristics with soils in high altitude being usually less well developed than lowland soils [233]. A study conducted in flowering heads of *Arnica montana* cv. ARBO reported a significant increase of caffeic acid derivatives with the altitude of the growing site [234].

P-I and P-II are the dynamic mediators accountable for the therapeutic effects of *P. kurroa,* and the disparity in the content of these compounds in plants at different elevations is a major question to be addressed. The P-I and P-II contents in various plant parts of *P. kurroa* collected from different altitudes in the North-Western Himalayas was analyzed by HPLC. A substantial degree of discrepancy in picroside content was detected [222]. Similar observations were made by Sharma *et al.* [2012] where picrosides content was maximum in the accession collected from the highest altitude from all other accessions. It was reported that picrosides content increased by 135% in the plants growing at high as compared to the low altitude [235] as an increase in altitude escorts a reduction in temperature and upsurge in light quanta [236].

2.11 Molecular basis of picrosides biosynthesis

Picrosides are monoterpene glycosides resulting from cyclization of geranyl pyrophosphate (GPP) to the iridoid moiety. Glucose and cinnamate/vanillate change iridoid into picroside-I and -II [237]. Conferring to the isoprene rule documented by Wallach and Rutzicka in the late nineteenth and mid-twentieth centuries [238], all terpenoids are resultant from the common five-carbon structure blocks, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). Efficacious manufacturing of terpenoid products in plants disparagingly rest on the flux of precursors transported by the essential isoprenoid biosynthetic pathways and, subsequently, on the active regulation of these biosynthetic routes. Plants employ two sovereign pathways to yield IPP and DMAPP: the principally cytosolic mevalonic acid (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway. The MVA pathway largely delivers the precursors for the cytosolic biosynthesis of sesquiterpenoids, polyprenols, phytosterols, brassinosteroids, and triterpenoids, and for terpenoid biosynthesis in mitochondria (e.g., ubiquinones, polyprenols), and the five-carbon units derived from the MEP pathway are rather used for the biosynthesis of hemiterpenoids (e.g., isoprene), monoterpenoids, diterpenoids, carotenoids and their breakdown products like chlorophyll, tocopherols, cytokinins, plastoquinones and gibberellins [239].

In addition to the transcriptional regulation of MVA and MEP pathway genes and their dissimilar paralogues, isoprenoid pathway fluxes are regulated at posttranscriptional/-translational levels and by feedback regulation. Very restricted

efforts have been made so far in understanding the molecular biology behind the biosynthesis of picrosides in *P. kurroa*, together with identification of genes/enzymes and metabolic pathways. Even though efforts have been made in understanding the molecular basis of picroside biosynthesis on transcriptomics basis, no proteome analysis has been done till date allied with the biosynthesis of picrosides in *P. kurroa*. Conferring to a projected strategy, metabolites, proteins and transcriptional profiling under two physiological states (for example, metabolites accumulating versus non-accumulating) can provide a fresh approach for pathway elucidation in plants [85]. As such differential proteome analysis between metabolites accumulating versus non-accumulating conditions could aid in identifying proteins involved in the biosynthesis of secondary metabolites such as picrosides.

Though transcriptomics investigation can make available a prodigious wealth of information about countless biological processes but it is partial without proteome analysis which supports in the enhanced understanding of events stirring inside a cell [240]. As the central dogma decrees the transcription of DNA to mRNA and the translation of mRNA to proteins, one would assume to find a connection between mRNA and protein abundances. But this is not factual, exploration has found the association between mRNA and protein to be meagre [87, 89].

Three explanations have been alleged for this meagre correlation; a) Momentous difference in the half-lives of proteins, b) Incapability to get a rich picture because of a substantial volume of error and noise in both protein and mRNA experiments and c) Several byzantine post-translational adjustments [88]. It has been pragmatic that DNA sequence and mRNA expression studies flop to deliver evidence concerning protein post-translational modification, structure and protein-protein interactions. Meant for carrying out various functions, virtually all proteins undertake post-translational modification, henceforth, it becomes vital to scrutinize the protein content so as to get an improved understanding of the innumerable physiological processes [90].

One more imperative reason which dictates the study of *P. kurroa* proteins is that proteomic studies to date have been carried out principally in model plants such as *Arabidopsis thaliana*, *Oryza sativa* (rice), *Populus trichocarpa* (black cottonwood), and *Vitis vinifera* (grapevine) for which copiously sequenced genomes are accessible. Very insufficient studies have been steered in the direction of biosynthesis

of secondary metabolites in medicinal plants expressly with the emphasis on recognizing novel enzymes involved in secondary metabolism [91]. One of these studies entails a proteomic tactic commenced for identification of new proteins involved in the alkaloid biosynthesis of *Catharanthus roseu*s. The study recognized strictosidine synthase (EC 4.3.3.2), which catalyzes the creation of strictosidine in the alkaloid biosynthesis; tryptophan synthase (EC 4.1.1.28), which is desired for the supply of the alkaloid precursor tryptamine and 12-ox-ophytodienoate reductase, which is circuitously involved in the alkaloid biosynthesis as it catalyzes the last step in the biosynthesis of the controller jasmonic acid [241]. In another study involving elicitor-induced PTOX accumulation in *P. hexandrum* cell cultures, an effort was made to reconnoiter protein profile of elicited cell suspension culture of *P. hexandrum* ensuing in boosted accumulation of PTOX. Using 2-DE a total of 233 spots stood noticed, out of which 105 spots were identified by MALDI TOF-TOF MS/MS. Several phenylpropanoid and monolignol pathway enzymes were identified, amongst which, chalcone synthase, polyphenol oxidase, caffeoyl CoA 3-O-methyltransferase, S-adenosyl-L-methionine dependent methyltransferases, caffeic acid-O-methyl transferase etc. were recognized as the most imperative. Other differentially hoarded proteins were identified to be involved in stress/defense-related response, transcription/DNA replication and signalling [91].

Picrosides are biosynthesized by the involvement of the mevalonate (MVA), non-mevalonate (MEP), iridoid and phenylpropanoid pathways.

The MVA pathway in plants comprises of six stages and starts with the Claisen-type condensation of two molecules of acetyl-CoA to acetoacetyl-CoA by acetoacetyl-CoA thiolase (AACT). In a following aldol condensation reaction catalyzed by HMG-CoA synthase (HMGS), AcAc-CoA is joint with a third molecule of acetyl-CoA to form the C6-compound S-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). In the next rate-limiting step, HMG-CoA reductase (HMGR) converts S-HMG-CoA to R-mevalonate in two NADPH-dependent reduction steps. MVA produced by HMGR is eventually transformed to IPP by a three-step enzymatic process: two ATP-dependent phosphorylation steps, catalyzed by mevalonate kinase (MK) and phosphomevalonate kinase (PMK), and an ATP-driven decarboxylative purging catalyzed by mevalonate diphosphate decarboxylase (MVD or MPDC). The MEP pathway is made up of seven

enzymatic steps. In the first reaction, 1-deoxy-D-xylulose 5-phosphate (DXP) is formed by DXP synthase (DXS) from (hydroxyethyl) thiamine diphosphate, which is derived from pyruvate, and glyceraldehyde-3-phosphate (GAP) in a transketolase like condensation. The enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) catalyzes the second step of the MEP pathway, in which DXP is transformed into 2-C-methyl-D-erythritol 4-phosphate (MEP) by an intramolecular rearrangement of DXP into 2-C-methyl-D-erythrose 4-phosphate, followed by a NADPH-dependent reduction [242, 243]. The reaction can be specifically inhibited by fosmidomycin, a structural analog of the DXPR substrate [244-246] thus barricading the biosynthesis of downstream plastidial terpene biosynthesis [247-249]. The reaction catalyzed by DXPR is in some cases well thought-out to stay a rate-limiting step depending on the species, tissue, and developmental stage. MEP is further converted in a CTP-dependent reaction to 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) by the enzyme 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (MCT or IpsD) [250, 251]. Phosphorylation of CDP-ME by the enzyme 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK, IspE) then leads to the formation of 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate (CDP-ME2P) [252], which is later cyclized by 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS, IspF) into 2-C-methyl-D-erythritol 2,4-cyclodi-phosphate (MEcPP) upon loss of CMP. In the last two steps of the MEP pathway, the enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS, IspG) initially transforms MEcPP in a two-electron reduction to 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP). In the ultimate diverging step, HMBPP is converted by 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR, IspH) to a collection of IPP and DMAPP with a ratio of 5 to 6:1 [253].

A few efforts have been made in understanding the molecular basis of picroside biosynthesis by cloning of genes and transcriptome sequencing followed by analysis using Illumina sequencing technology. Kawoosa et al. 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 *P. kurroa* for studying light and temperature responsiveness implying 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 ± 1 °C) and under illumination as compared

to 25 ± 1 °C and dark conditions [54]. This established a correlation between picroside content and gene expression as the quantity of picroside was also found to be raised at 15 ± 1 °C as compared to 25 ± 1 °C. In another study transcriptome sequencing and analyses was carried out using plants grown at two temperature conditions 15 ± 1 °C and 25 ± 1 °C to gain a better insight into the temperature-mediated molecular changes. Expression profiling revealed amendments in numerous biological processes and metabolic pathways including cytochrome P450s (CYPs), UDP-glycosyltransferases (UGTs) and those related with picrosides biosynthesis. Augmented picrosides content and upregulation of CYPs and UGTs at 15 ± 1 °C advocated these to be the probable applicants associated with picrosides biosynthesis [254]. Work done by Singh et al. advanced the understanding on the molecular basis of picrosides biosynthesis. Eight full-length cDNA sequences from MEP and MVA pathway genes and two partial sequences from phenylpropanoid pathway were cloned followed by expression analysis regarding picrosides content in different tissues. The expression of these genes was detected to be the extreme in leaf tissue followed by rhizome and root which was in congruence with the identified picroside-I level in these tissues [255]. Work done by Pandit et al., 2013 further contributed to the understanding molecular biology of picroside biosynthesis. Five genes of MEP and MVA pathway were cloned using comparative genomics trailed by expression investigation of all 15 genes of these pathways done concerning picrosides level in diverse tissues and developmental stages of *P. kurroa*. Several genes exhibited elevated expression level of transcripts in different tissues. Four genes of MEP pathway and one gene of MVA pathway exhibited elevated levels of transcripts in shoots and stolons, four genes of MEP pathway showed elevated expression in roots. There was only one gene which displayed higher expression in shoots as well as roots. None of the genes showed elevated expression in rhizome tissue suggesting its role only as a storage organ for picrosides. The study identified vital genes involved in the biosynthesis of GPP, the precursor for picrosides biosynthesis [71].

Despite several attempts, the complete biosynthetic pathway for picrosides remained obscured. A plausible pathway for the biosynthesis of picrosides was given by Kumar et al. [52] using a bio-retrosynthetic approach. In this conceptual approach, the researcher begins by considering the structure and properties of a target molecule. Then, on the basis of knowledge of the enormous body of known chemical transformations, the researcher

identifies a precursor(s) of the target molecule that, upon a given chemical transformation, will yield the target molecule. The precursor(s) identified by this method can then become the starting point for another retrosynthetic step. That is, the researcher can identify a precursor for the precursor (a penultimate precursor) used in the subsequent step. This process is repeated until the researcher ultimately finds a suitable starting material from which to begin the synthesis of the target product [256]. This can be achieved by literature survey and fragmentation or dissection of the target compound. The dissection of target compound results in smaller daughter compounds which might be participating in the biosynthesis of the target compound. Care should be taken that fragmentation of compounds is done according to a set of predefined 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. The intermediates obtained were then validated using LC-MS-MS approach which involved developing ways for separation of those intermediates. The intermediates were further confirmed by means of fragments matching or proposed fragmentation. The presence of different intermediates for the first time in *P. kurroa* were reported using LC/ESI–MS/MS method. The method confirmed the occurrence of catalpol and aucubin, the major backbone structures of picrosides, along with transitional metabolites boschnaloside, bartsioside and mussaenosidic acid. Shitiz et al., 2015 based on the transcriptome data further proposed the enzymes catalyzing the various steps in the picroside biosynthetic pathway. Initially, the study predicted enzyme class on the basis of the chemical reaction/group transfer. KEGG database was then searched to identify enzymes catalyzing similar type of reactions. The enzyme sequences obtained were then matched to *P. kurroa* transcriptome and the enzymes involved in metabolite biosynthesis were shortlisted. The enzymes were then further shortlisted based on transcript richness in transcriptomes. The elevated expression of gene transcripts for corresponding enzymes concerned with P-I and P-II contents and their participation in secondary metabolism in other plant species advocated their potential role in catalyzing the required enzymatic reactions in picrosides biosynthesis [72]. However, identification and validation of proteins associated with picroside biosynthesis remained elusive.

Primary metabolic pathways such as photosynthesis, glycolysis, sucrose/starch synthesis pathway, TCA/Citric acid cycle, etc. often act as feeder pathways providing precursors for

secondary metabolic pathways. Therefore, an appropriate understanding of picrosides biosynthesis necessitates the synchronization of primary and secondary metabolic pathways. 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* was studied with the objective of identifying regulatory steps that provides insight into the metabolic basis controlling the biosynthesis of picroside-I. The study revealed that HK, DXPS, ISPD, HMGR and PMK genes associated with primary as well as secondary metabolic pathways contributed to picroside-I biosynthesis for the initial 20 days of plant growth establishing the significant role of MEP pathway as compared to the MVA pathway. Similarly, between 20-30 days of *P. kurroa* growth *in vitro*, increased expression of DAHPS and G10H related to shikimate/phenylpropanoid and iridoid pathway, respectively indicated their potential role in picroside-I biosynthesis suggesting them as the conceivable regulators of picroside-I biosynthesis [257].

CHAPTER 3
MATERIALS AND METHODS

3.1 Plant samples

3.1.1 For differential protein expression studies in P. kurroa to identify proteins involved in the biosynthesis of picroside-I and increased shoot biomass

Field grown plantlets of *P. kurroa* were collected from nursery (Jagatsukh, Himachal Pradesh; 32° 12' 0N, 77° 12' 0E; 2193 m altitude). The shoot apices collected from field grown plants were surface sterilized and cultured in an optimized Murashige and Skoog (MS) medium supplemented with 3mg/L indole-3-butyric acid and 1mg/L kinetin at Jaypee University of Information Technology (Waknaghat, Himachal Pradesh; 31°0' 58.55" N, 77.4' 12.63" E; 1700 m altitude). These cultures were nurtured in tissue culture chambers at a different temperature of 15 ± 1 °C and 25 ± 1 °C with a 16 h photoperiod provided by cool white fluorescent light (3,000 lux) [258]. These cultures were grown for 4–5 weeks and samples were collected. The collected samples were separated into different parts *viz*. shoots, roots and immediately stored at −80 °C until further use. Shoots were selected for differential protein expression studies on the basis of ~17 times higher picroside-I content and enhanced shoot biomass at 15 ± 1 °C *vis-à-vis* at 25 ± 1 °C (Figure 3.1) [259].

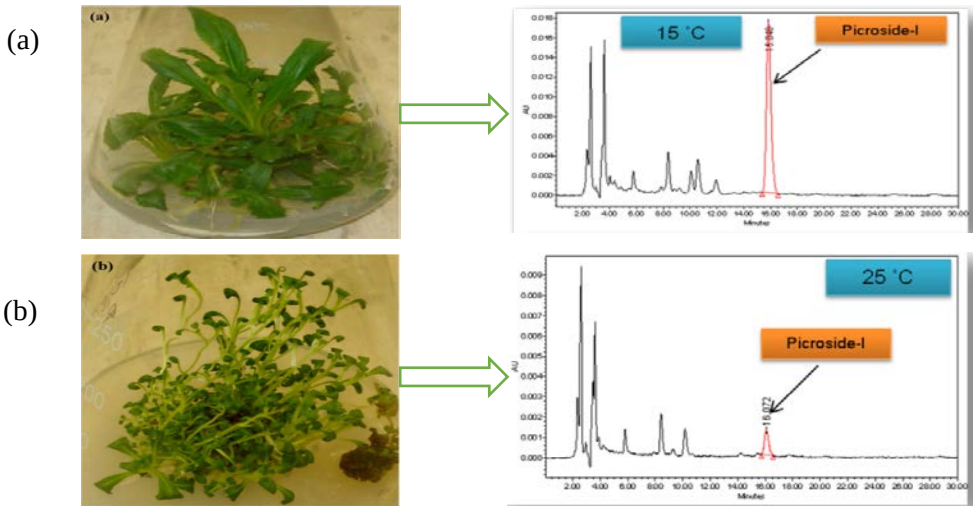


Figure 3.1 Enhanced shoot biomass and picroside-I levels in tissue cultured *P. kurroa* maintained at (a) 15 ± 1 °C as compared to (b) 25 ± 1 °C [259]

3.1.2 For differential protein expression studies in P. kurroa to investigate the presence of proteins involved in the biosynthesis of picroside-I and picroside-II in stolons.

Roots were obtained from *P. kurroa* plants maintained by tissue culture at 15 ± 1 °C and stolon samples were taken from *P. kurroa* obtained from Sairopa (4,500 m altitude, 31°38'–31°54'N and 77°20'–77°45' E) with respect to differential picroside-I & II content.

3.2 Optimization of protein extraction method

In order to optimize protein extraction for maximum protein yield, various extraction protocols were used to find out the extraction method that would give the highest amount of protein yield. The samples were grounded using liquid nitrogen and subjected to different extraction methods. The extraction methods that were used in the optimization are as follow:

3.2.1 Tris-Cl extraction method

0.4 g of powdered samples of various tissues were taken separately and extracted with 2 mL of 50 mM Tris-Cl buffer (pH 7.4) containing 0.25 M NaCl, 0.07% sodium azide for 24 h with gentle stirring at 4°C. The slurry thus obtained was centrifuged at 10,000 x g for 20 min at 4°C to recover the supernatant. The supernatant was collected and stored as aliquots at -80°C [260].

3.2.2 Phosphate buffer extraction method

Phosphate extraction buffer consisting of 0.2 M phosphate buffer (pH 7.2), 150 mM sodium chloride, 8 M urea, 1% (w/v) CHAPS, 10% (v/v) glycerol and 2 mM EDTA was added to 1 g powdered tissue in a ratio of 3:1. The mixture was at that point centrifuged at 14,000 x g for 25 min at 18 °C. The supernatant was recovered and stored as aliquots at -80°C [261].

3.2.3 TCA-Acetone extraction method

1 g tissue sample was homogenized in 10 mL of cold acetone (-20°C) containing 10% (w/v) trichloroacetic acid (TCA) and 0.07% (v/v) 2-mercaptoethanol (2-ME) and 0.1 g of water-insoluble polyvinylpyrrolidone (PVP). The mixture was kept at -20°C for 1 h, and then centrifuged at 13,000 x g for 20 min at 4°C. The pellet was washed twice

with 80% cold acetone and precipitated by adding 4 volumes of cold acetone. Finally, the white pellet was lyophilized and stored at -80°C [262].

3.2.4 Water extraction method

1 g tissue sample was agitated in 2-3 ml of distilled water on a magnetic stirrer at 4°C for 24 h and then centrifuged at 10,000g for 20 min at 4°C. The supernatant was collected and stored as aliquots at -80°C.

3.3 Protein Estimation

3.3.1 Lowry's protein estimation

The amount of protein obtained from each of the extraction methods was estimated using Lowry's protein estimation which is a biochemical assay for determining the total level of protein in a solution. The method involves the reactions of copper ions with the peptide bonds under alkaline conditions resulting in the oxidation of aromatic protein residues. The Lowry method is based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin–Ciocalteu reaction). The reaction mechanism is not well understood but involves reduction of the Folin–Ciocalteu reagent and oxidation of aromatic residues (mainly tryptophan, also tyrosine). Experiments have shown that cysteine is also reactive to the reagent. Therefore, cysteine residues in protein probably also contribute to the absorbance seen in the Lowry Assay [263]. Total protein concentration is exhibited by a color change of sample solution in proportion to the protein concentration which can then be measured using colorimeter technique.

Reagents: -

1. Lowry A: 2% Na_2CO_3 in 0.1 N NaOH
2. Lowry B: 1% $CuSO_4$ in Distilled Water
3. Lowry C: 2% Sodium Potassium Tartrate
4. Lowry stock reagent: 49 mL A + 0.5 mL B + 0.5 mL C
5. Folin–Ciocalteu reagent: Folin Reagent (2N): Distilled Water = 1:1
6. Standard Stock Solution of BSA of concentration of 2mg/ml

The methodology followed for protein estimation using Lowry's protein estimation method is summarized in Figure 3.2.

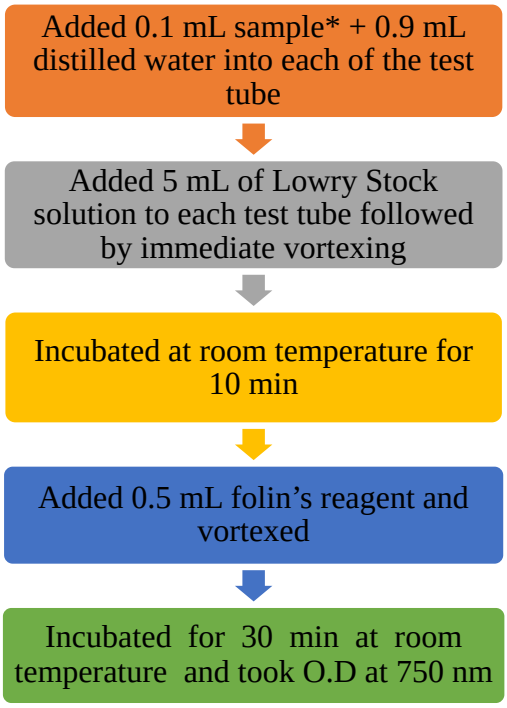


Figure 3.2 Workflow for protein concentration estimation using Lowry's protein estimation method (*sample refers to different concentrations of BSA in case of standard, distilled water/buffer in case of blank and extracted protein in case of unknown/test sample)

3.3.2 Bio-Rad RC DC protein assay

This assay is based on the Lowry assay but has been modified to be reducing agent compatible (RC) as well as detergent compatible (DC). Protein content in samples extracted in buffers containing reducing agents and detergents was estimated using this assay the methodology for which is summarized in Figure 3.3.

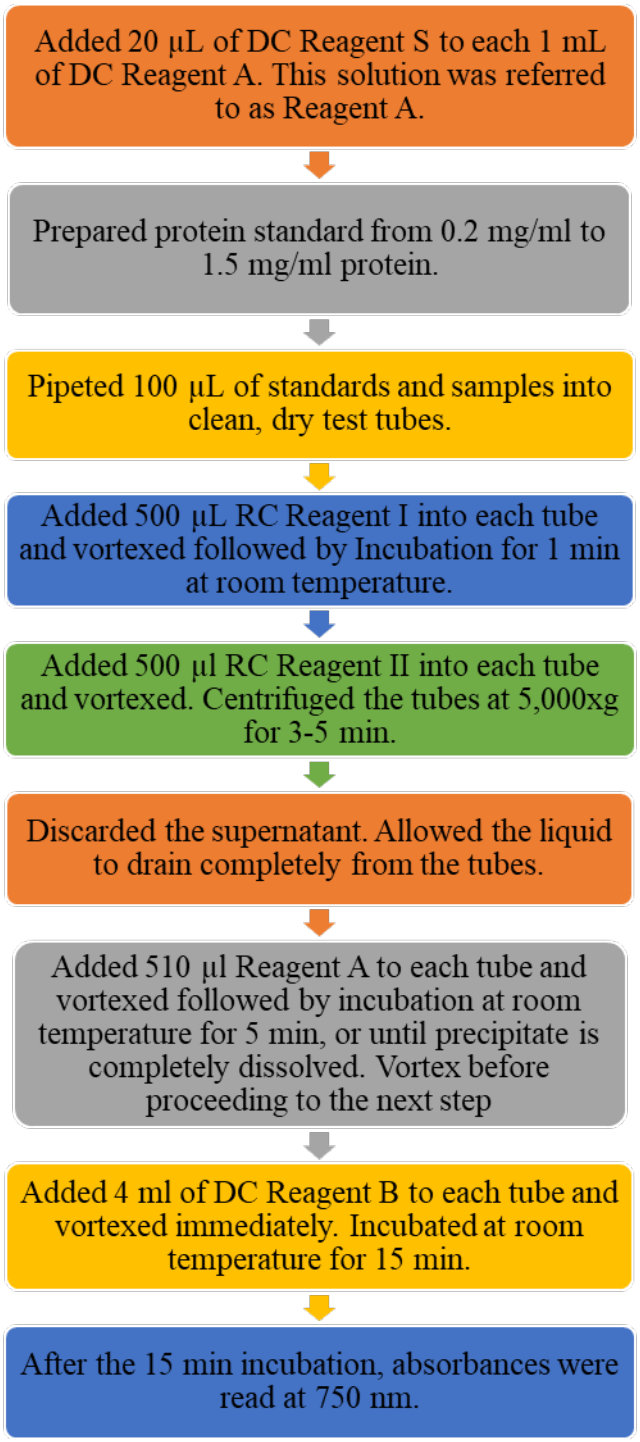


Figure 3.3 Workflow for protein concentration estimation using Bio-Rad RC DC protein assay

3.4 Protein Extraction

3.4.1 For differential protein expression studies in P. kurroa to identify proteins involved in the biosynthesis of picroside-I and increased shoot biomass

a) SDS-PAGE analysis

0.4 g of powdered shoot samples of both 15 ± 1 °C and 25 ± 1 °C maintained *P. kurroa* were taken separately and extracted with 2 mL of 50 mM Tris-Cl buffer (pH = 7.4) containing 0.25 M NaCl, 0.07% sodium azide for 24 h with gentle stirring at 4°C. The slurry thus obtained was centrifuged at 10,000g for 20 min at 4°C to recover the supernatant. The supernatant was collected and stored as aliquots at -80°C.

b) 2-D gel electrophoresis

15 ± 1 °C and 25 ± 1 °C shoot samples were washed with sterile water, dried and grounded to a fine powder using liquid nitrogen in a precooled pestle and mortar. 0.5 g of powdered shoot sample of both 15 ± 1 °C and 25 ± 1 °C maintained *P. kurroa* were taken separately and transferred to a polypropylene tube containing 5 mL of chilled (−20 °C) 10% (w/v) trichloroacetic acid (TCA) in 100% (v/v) acetone containing 0.07% (w/v) dithiothreitol (DTT) and vortexed. For comprehensive precipitation, samples were incubated overnight at -20°C, trailed by centrifugation at 15,557 x g for 45 min. The pellets were resuspended in 100% (v/v) acetone containing 0.07% DTT for 1 hour, followed by centrifugation at 15,557 x g for 45 min. This stage was repeated thrice to completely remove any residual TCA. The pellet was then air dried to remove acetone and resuspended in lysis buffer containing 7 M urea, 2 M Thiourea, 2% (w/v) CHAPS, 1% (w/v) DTT, 2% biolyte pH 3–10 and protease inhibitor cocktail. The samples were then sonicated in a water bath maintained at 20°C for 30 min followed by gentle stirring at room temperature for 4 h. The samples were centrifuged at 15,557 x g for 45 min and supernatant obtained was transferred to a new tube for further centrifugation at 15,557 x g for 45 min to eradicate any outstanding precipitate. The clear supernatant obtained was divided into aliquots and stored at -80°C. Protein concentration was estimated using the Bio-Rad RC DC™ protein estimation kit.

3.4.2 For differential protein expression studies in P. kurroa to investigate the presence of proteins involved in the biosynthesis of picroside-I and picroside-II in stolons

SDS-PAGE analysis

The washed and dried samples were frozen in liquid nitrogen and grounded in a precooled pestle and mortar to attain a fine powder. This fine powder was then suspended in 10% (w/v) trichloroacetic acid (TCA) in 100% (v/v) acetone comprising 0.07% (w/v) dithiothreitol (DTT). For comprehensive precipitation, samples were incubated overnight at -20°C, trailed by centrifugation at 15,557 x g for 45 min. The pellets were resuspended in 100% (v/v) acetone containing 0.07% DTT for 1 hour, followed by centrifugation at 15,557 x g for 45 min. This stage was repeated thrice to completely remove any residual TCA. The pellet was then air dried to remove acetone and resuspended in lysis buffer containing 7 M urea, 2 M Thiourea, 2% (w/v) CHAPS, 1% (w/v) DTT, 2% biolyte pH 3–10 and protease inhibitor cocktail. The samples were then sonicated in a water bath maintained at 20°C for 30 min followed by gentle stirring at room temperature for 4 h. The samples were centrifuged at 15,557 x g for 45 min and supernatant obtained was transferred to a new tube for further centrifugation at 15,557 x g for 45 min to eradicate any outstanding precipitate. The clear supernatant obtained was divided into aliquots and stored at -80°C. Protein concentration was estimated using the Bio-Rad RC DC™ protein estimation kit.

3.5 Protein separation

3.5.1 For differential protein expression studies in P. kurroa to identify proteins involved in the biosynthesis of picroside-I and increased shoot biomass

a) SDS-PAGE

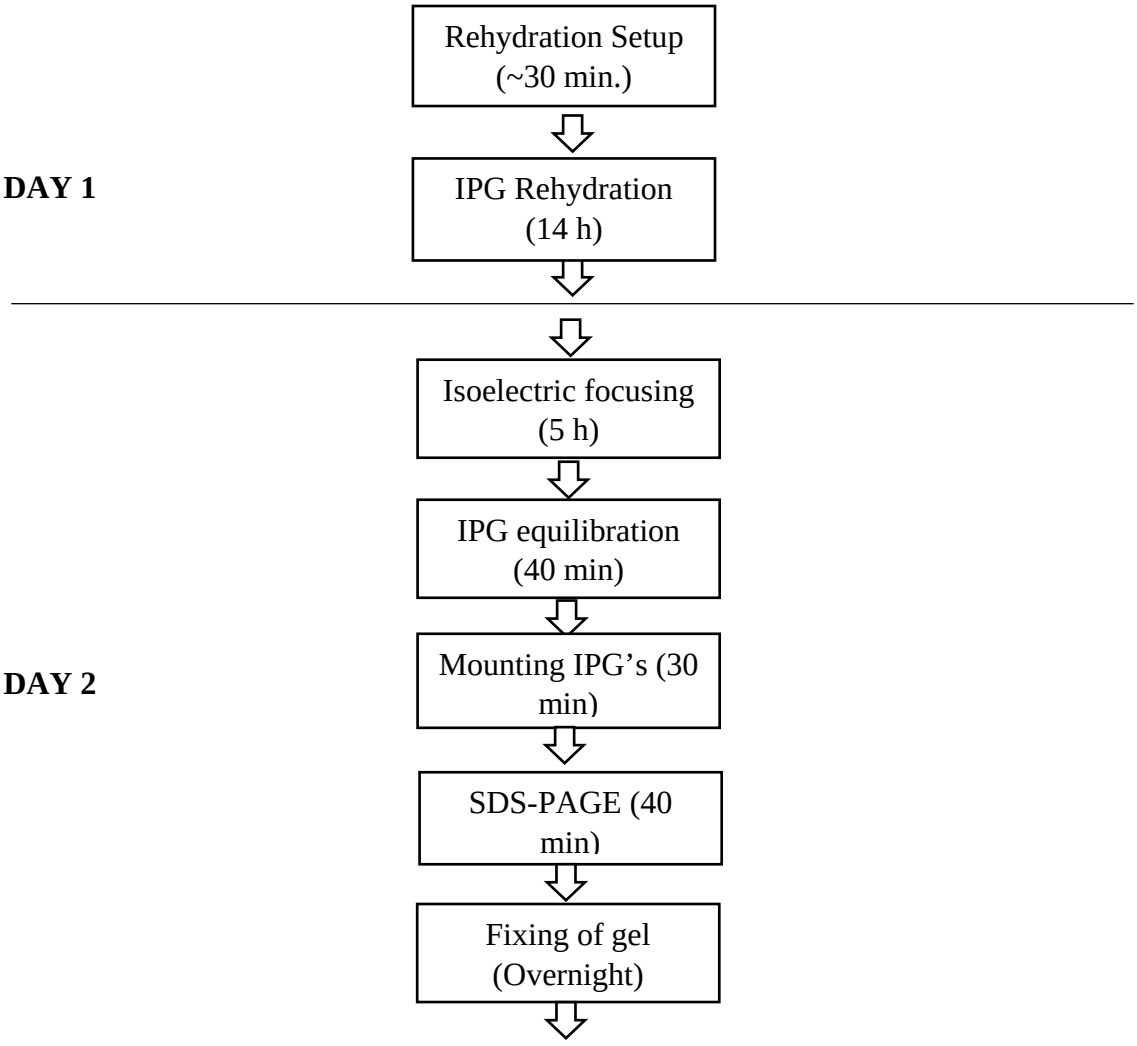
Protein content in the extracted samples was estimated using Lowry's protein estimation method. Samples extracted using Tris-Cl extraction method were desalted using Bio-Rad Micro Bio-spin 6 Columns and loaded onto a discontinuous gel system containing 12.5% resolving gel and 4% stacking gel prepared according to the recipe listed in Table 3.1. Separation was carried out at a constant voltage of 100V using a Bio-Rad PowerPac™ Universal power supply.

Table 3.1 Recipe for preparing stacking and resolving gels for SDS-PAGE

Resolving Gel	
Component	**For 10 mL**
Distilled H_2O	3.175 mL
30 % Acrylamide	4 mL
1.5 M Tris (pH = 8.8)	2.503 mL
10 % SDS	100 µL
10 % APS	100 µL
TEMED	4 µL

Stacking Gel	
Component	**For 4 mL**
Distilled H_2O	2.66 mL
30 % Acrylamide	670 µL
1M Tris (pH = 6.8)	500 µL
10 % SDS	40 µL
10 % APS	40 µL
TEMED	4 µL

b) 2-D gel electrophoresis



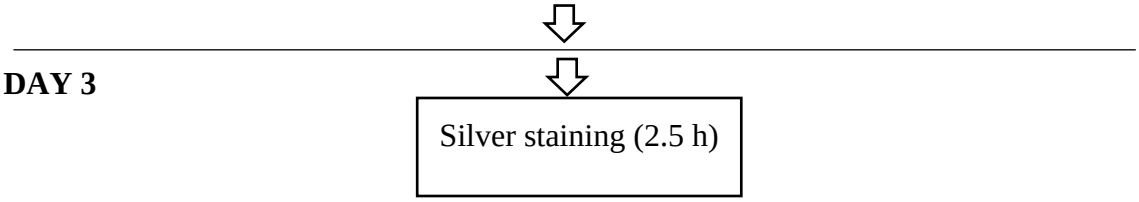


Figure 3.4 Workflow for 2-D gel electrophoresis

Sample Preparation

100 µg of protein from each of the samples was precipitated using chloroform-methanol precipitation method which is summarized in Figure 3.5.

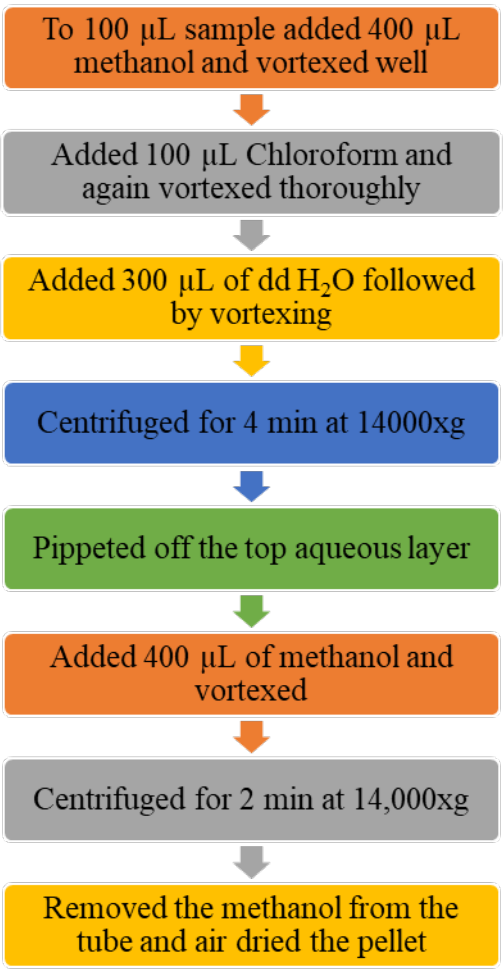


Figure 3.5 Workflow for sample preparation for performing 2-D gel electrophoresis

Rehydration of IPG strips

The air-dried pellet was resuspended in 125 µL of rehydration buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte® 3/10 ampholytes, and bromophenol Blue (trace)). The resuspended samples were then used to passively rehydrate 7 cm pH 3–10 immobilized pH gradient (IPG) strips (Bio-Rad, USA) for 14 hours at 20 °C. The required amount of protein sample was pipetted as a line along the back edge of a channel. The line of sample extended along the whole length of the channel except for about 1 cm at each end. It was made sure that no air bubbles were introduced during the as they would interfere with the even distribution of the sample in the strip. When all the protein samples had been loaded into the rehydration tray, using forceps, peel the cover sheet from one of the pH 5-8 Ready Strip IPG strips. Gently placed the strip gel side down onto the sample. The "+" and "pH 5-8" should be legible and situated at the eft side of the tray. Overlaid each of the strips with 2 to 3 mL of mineral oil to prevent evaporation during the rehydration process. The mineral oil was added slowly, by carefully dripping the oil against the plastic backing of the strips while moving the pipet along the length of the strip. Covered the rehydration tray with the plastic lid provided and left the tray sitting on a level bench overnight (14 h) to rehydrate the IPG strips and load the protein sample.

Isoelectric focusing (IEF)

IEF was performed on Bio-Rad Protean IEF Cell. Placed a clean, dry PROTEAN IEF focusing tray the same size as the rehydrating IPG strips onto the lab bench. By means of forceps, placed a paper wick at both ends of the channels covering the wire electrodes. Pipetted 8 µL of nanopure water onto each wick to wet them. Removed the cover from the rehydration tray containing the IPG strips. Via forceps, carefully held the strips vertically for about 7 to 8 seconds to allow the mineral oil to drain, then transferred the IPG strips to the corresponding channel in the focusing tray (maintain the gel side down). Covered each IPG strip with 2 to 3 ml of fresh mineral oil. A lid was placed onto the focusing tray and the tray was then placed into the PROTEAN IEF cell and the cover was closed. Programmed the PROTEAN IEF cell using the 3-step protocol listed in Table 3.2. For all strips, the default cell temperature of 20 ºC, with a maximum current of 50 µA/strip was used.

Table 3.2 Program fed into Bio-Rad Protean IEF cell for performing isoelectric focusing

	Voltage	**Time**	**Volt-Hours**	**Ramp**
Step 1	250	20 min	----------	Linear
Step 2	4000	2 h	----------	Linear
Step 3	4000	----------	10,000 V-h	Rapid
Total		5 h	14,000 V-h	

Equilibration of IPG strips

When the electrophoresis run completed, the IPG strips were removed from the focusing tray and transferred gel side up into a new or clean, dry disposable rehydration tray. The strips were held vertically for a few seconds with the help of forceps to drain mineral oil. Added 2.5 mL of equilibration buffer 1 (6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 2% (w/v) DTT) to each channel containing an IPG strip. The tray was placed on an orbital shaker and gently shaken for 20 min. At the end of 20 min incubation, the strips were lifted with the help of forceps and placed into fresh channels. 2.5 mL of equilibration buffer 2 (6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol and 135 mM iodoacetamide) was added to each channel. The tray was returned to the orbital shaker for another 20 min. During the incubation, melt the overlay agarose solution (0.5% low melting point agarose in 25 mM Tris, 192 mM glycine, 0.1% SDS, and a trace of Bromophenol Blue) in a microwave. At the end of the 20 min incubation period, the equilibration buffer 2 was decanted from all the channels.

SDS-PAGE

Prepare 12 % SDS-PAGE gels as described above but without the stacking gel. Filled a 100-mL graduated cylinder with 1X Tris-glycine-SDS running buffer.

Removed an IPG strip from the disposable rehydration tray and dipped briefly into the graduated cylinder containing the 1X Tris/glycine/SDS running buffer. Laid the strip gel side up and onto the back plate of the SDS-PAGE gel above the IPG well. Pipetted overlay agarose solution into the IPG well of the gel. Using the forceps, carefully

pushed the strip into the well. Placed the gels vertically and allowed the agarose to solidify for 5 min. After solidification of agarose, SDS-gels were run at a constant voltage of 100V until the dye front reached the bottom of the gel. Gels were fixed overnight in a fixing solution consisting of 40% methanol, 10 % acetic acid and 50 % distilled water following which they were silver stained.

3.5.2 For differential protein expression studies in P. kurroa to investigate the presence of proteins involved in the biosynthesis of picroside-I and picroside-II in stolons

SDS-PAGE analysis

The required amount of sample was precipitated by adding 100 % (v/v) acetone containing 0.07 % DTT in 1:4 ratio. The samples were kept at -20°C for at least 2 hours, followed by centrifugation at 20,000 x g for 15 min. The pellet obtained was air dried at room temperature and resuspended in 2x Laemmlli buffer. The samples were loaded onto a discontinuous gel system containing 12 % resolving gel and 5 % stacking gel. Separation was carried out using Bio-Rad PROTEAN II xi cell at 16 mA for first 30 min, followed by 24 mA till dye front reached the bottom of the gel. The gels were visualized by silver staining. SDS-PAGE gel separations were repeated for a total of 3 times.

3.6 Gel staining

To visualize the separated proteins, the gels were stained after protein separation using silver staining technique. Amongst the various protein visualization methods available, silver staining has gained extensive popularity owing to its sensitivity (in the very low ng range), can be performed with simple and cheap laboratory reagents, and does not require complicated and expensive hardware for the readout. The rationale of silver staining is quite simple. Proteins bind silver ions, which can be condensed under appropriate conditions to build up a visible image made of finely divided silver metal [264]. The methodology used for silver staining is summarized in Figure 3.6

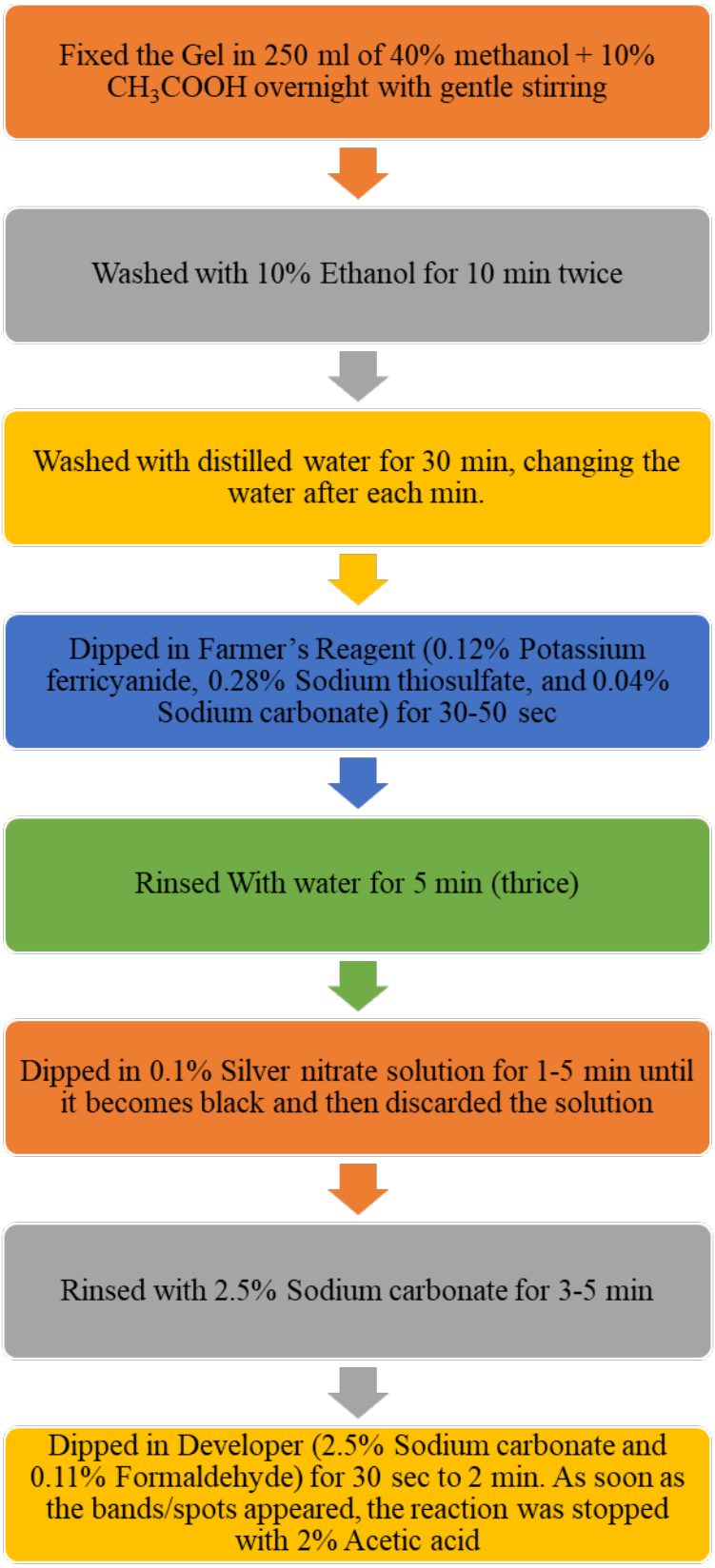


Figure 3.6 Methodology followed for silver staining of SDS-PAGE and 2-D gel electrophoresis gels

3.7 Image acquisition and analysis

3.7.1 For SDS-PAGE gels

The gels were visualized using silver staining. Gels were scanned at a resolution of 36.3 X 36.3 microns using Bio-Rad GS-800 Calibrated Densitometer and image analysis was performed using Bio-Rad's Quantity One software.

3.7.2 For 2-D gel electrophoresis gels

The silver stained gels were scanned as described above. All mage analysis and differential studies were performed using Bio-Rad's PD Quest advanced 2-D analysis software. The spots were initially detected in auto detection mode and then finalized using manual identification to remove artifacts and add undetected spots. Landmark identification tool of the software helped in the matching of spots across all gels. A local regression model of normalization method was used to normalize spot intensities against the total intensity of the gel. The differentially expressed spots that were reproducibly detected were selected for MALDI-TOF MS analysis.

3.8 MALDI-TOF MS analysis

The differentially expressed spots identified after the image analysis were excised and cut into small pieces of approximately 1 mm followed by destaining of the pieces in a freshly prepared 1:1 (v/v) mixture of 30mM potassium ferricyanide and 100 mM sodium thiosulphate. The supernatant was extracted and discarded followed by addition of 100 % acetonitrile. The mixture was incubated till the gel pieces shrank and turned white. Acetonitrile was removed, and gel pieces were completely dried. The gel pieces were then incubated with 100 – 200 µl of 10 mM dithiothreitol in 100mM ammonium bicarbonate for 1 h at 56 °C. The gel pieces were cooled to room temperature and the DTT solution was discarded. Next, added 100 – 200 µl of 55 mM (10mg/mL) iodoacetamide in 100 mM ammonium bicarbonate and incubated in dark for 45 min. The supernatant was removed, and the gel pieces were incubated in 100 mM ammonium bicarbonate solution for 5 minutes at room temperature. After removing and discarding the solution, the gel pieces were dehydrated with 100 µl of 100% acetonitrile for 10 min and Speedvac till complete dryness. Repeat the above two steps again. 20 µl (0.4 µg of trypsin) of the trypsin solution, prepared by adding 100 µl of 1 mM HCl, and 900 µl of 40 mM ammonium bicarbonate to one 20 µg vial of trypsin (Sigma), was added to the gel pieces and incubated at 37 °C

overnight (16 – 18 h. Peptides were eluted and reextracted in 50 % acetonitrile (ACN) containing 1 % trifluoroacetic acid (TFA). The samples were purified using ZipTip and mixed with α-cyano-4-hydroxycinnamic acid (4-*HCCA*) matrix in 1:1 ratio followed by plating onto a MALDI plate. After air drying, the plate was evaluated using MALDI-TOF/TOF ultraflex III instrument and further analysis was done with flex analysis software for obtaining the peptide mass fingerprint.

3.9 MASCOT protein identification

The peptide mass fingerprint data obtained from MALDI-TOF/TOF MS analysis was used to identify proteins using the MASCOT protein database search engine upheld at http://www.matrixscience.com. Peptides were presumed to be monoisotopic, carbamidomethylated at cysteine residues, and oxidized at methionine residues. Only 1 maximal cleavage was allowed for peptide matching and mass tolerance was varied between 100 ppm and 1.2 Da. Swissprot and NCBInr databases were searched with Viridiplantae and other green plants as the preferred taxonomies (Figure 3.7).

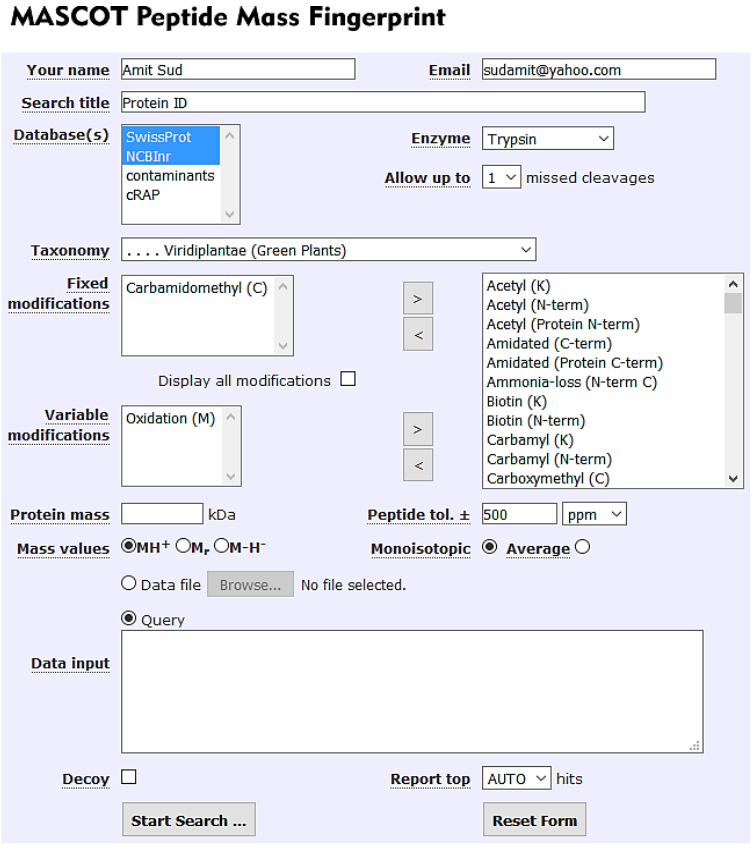


Figure 3.7 Mascot search engine interface

3.10 Western Blot

This technique is used to establish the specificity of antibodies and their ability to recognize components present in a complex mixture. In this method, it is necessary to initially separate the antigenic components using SDS-PAGE. It commonly involves the transferring of separated proteins from polyacrylamide gels to porous membrane and probing this blot with the antibody. Antibody-antigen complexes are then detected using enzyme labeled anti-immunoglobulins. Antigens recognized by specific antibody thus appear as bands on substrate developed blots.

3.10.1 SDS-PAGE

Samples extracted using Tris-Cl extraction method were desalted using Bio-Rad Micro Bio-spin 6 Columns and loaded onto a discontinuous gel system containing 12.5% resolving gel and 4% stacking gel. Separation was carried out in a Bio-Rad Mini-PROTEAN® Tetra Cell at a constant voltage of 100V as described before.

3.10.2 Protein transfer

Whatman paper was cut per the size of the gel. Soaked the filter paper in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and placed the first filter paper on transfer apparatus. Placed the second filter paper and removed any trapped air bubbles by rolling a glass pipette over it. Placed the third filter paper and again removed trapped air bubbles. Placed pre-wetted membrane on the filter paper and removed any air bubbles with the help of a pipette as described above. Placed the gel on the membrane. Placed another filter paper on top and removed air bubbles as described earlier. Placed the additional two filter papers on top as described earlier. Poured some buffer on top of each layer of the sandwich to fully wet the paper and removed excess buffer that had spilled. Connected the power pack and ran at 2 mA/cm^2 for 1 h. The transfer efficiency was checked by staining the membrane with Ponceau S stain.

3.10.3 Immunoblotting

Removed the membrane from the sandwich after completion of transfer. Washed the membrane 5 times with PBST (1X PBS with 0.05% Tween-20) for 5 min each. Blocked PVDF membrane with 5% skim milk in PBST. Washed membrane 3 times with PBST for 5 min each. Probed the membrane with 1:500 dilution of anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Abcam, ab125247) in 5% skim milk in PBST for overnight at 4

°C. Washed membrane 3 times in PBST for 5 min each. Probed the membrane with 1:1000 dilution of Anti-mouse IgG alkaline phosphatase conjugated antibody (Abcam, ab7077) prepared in 5% skimmed milk in PBST for 1 h at room temperature. Washed the membrane 3 times with PBST for 5 min each. Added substrate (10 mL alkaline phosphate buffer, 33 µL NBT, 16.5 µL BCIP) to the PVDF membrane. As soon as colour develops, stop the reaction by rinsing with water.

3.10.4 Image acquisition and analysis

The membrane was scanned at a resolution of 36.3 X 36.3 microns using Bio-Rad GS-800 Calibrated Densitometer and image analysis was performed using Bio-Rad's Quantity One software.

CHAPTER 4

RESULTS

4.1 Optimization of protein extraction method

The amount of protein in all the samples extracted using various extraction methods was estimated using Lowry's protein estimation method. The amount of protein obtained from different tissues using various extraction methods is shown in Figure 4.1, 4.2 and 4.3. For shoots and roots, the highest protein concentration was obtained by Tris-Cl extraction method followed by Phosphate buffer extraction method and then by TCA-Acetone extraction method. In case of stolons highest protein yield was obtained by TCA/Acetone method, followed by phosphate buffer and then Tris-Cl extraction method. As expected the lowest protein concentration was obtained using water extraction method which did not contain any reagent to increase protein solubilization.

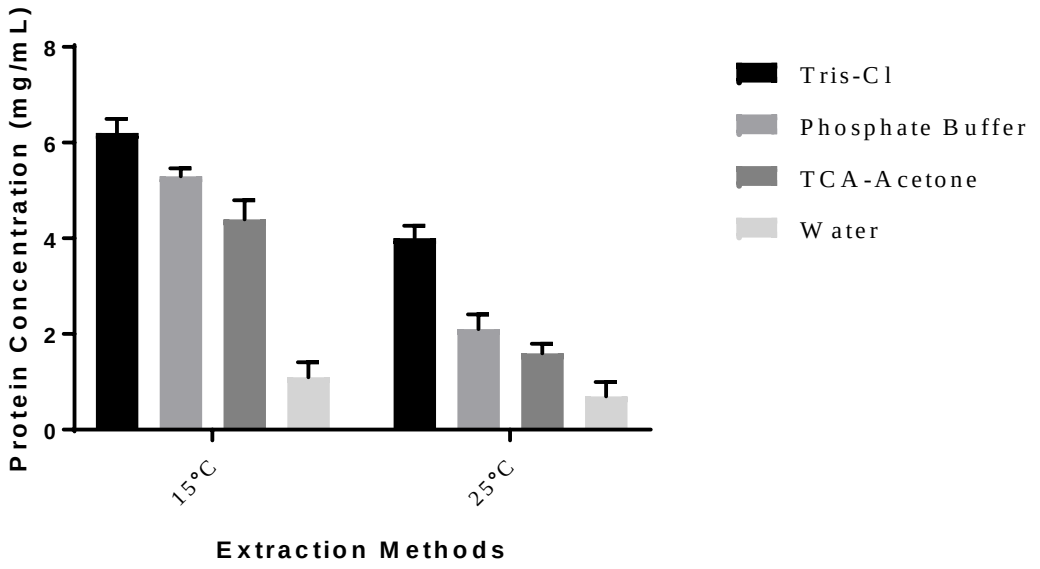


Figure 4.1 Protein concentration obtained from shoots of 15 ± 1 °C and 25 ± 1 °C *P. kurroa*

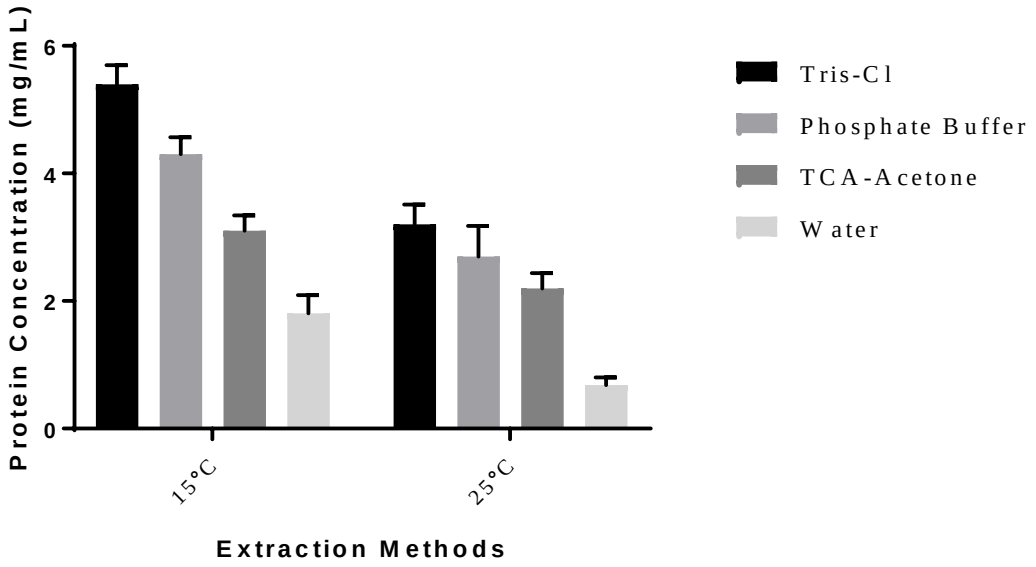


Figure 4.2 Protein concentration obtained from roots of 15 ± 1 °C and 25 ± 1 °C *P. kurroa*

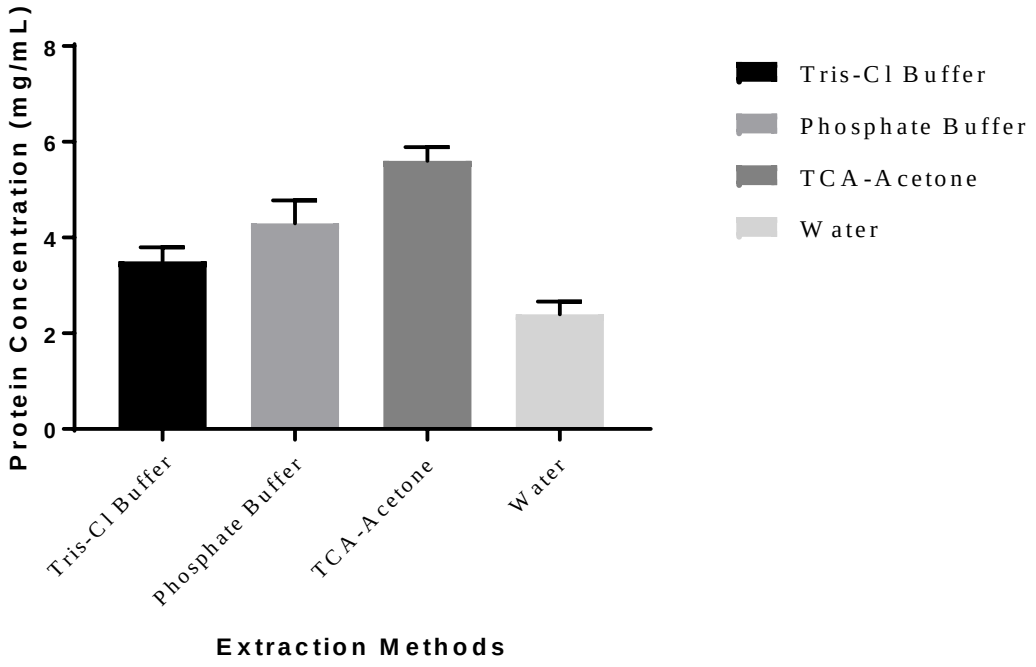


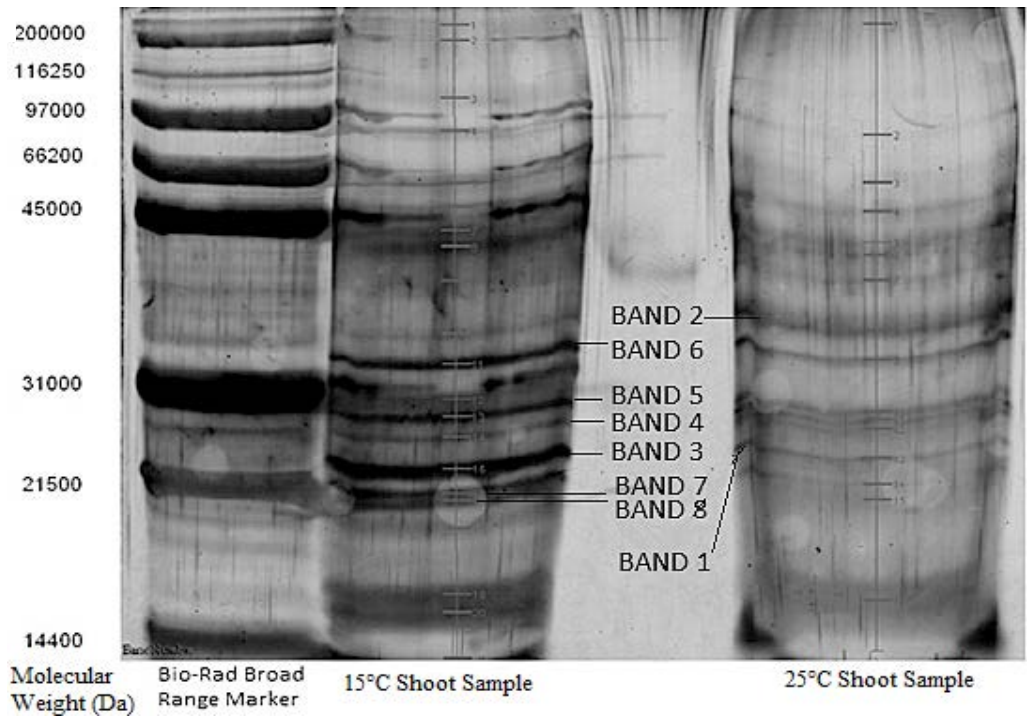
Figure 4.3 Protein concentration obtained from stolons of *P. kurroa* obtained from Sairopa

4.2 Differential protein expression studies in *P. kurroa* to identify proteins involved in the biosynthesis of picroside-I and increased shoot biomass.

a) SDS-PAGE analysis

Figure 4.4 (a) shows differential protein expression profile of 15 ± 1 °C shoot and 25 ± 1 °C shoots of *P. kurroa* with 21 bands and 16 bands in case of 15 ± 1 °C and 25 ± 1 °C shoot samples respectively (determined by Bio-Rad Quantity One software). In case of 15 ± 1 °C shoot samples, the number, as well as the intensity of protein bands, is higher as compared to 25 ± 1 °C shoot samples as confirmed by densitogram analysis performed using Bio-Rad Quantity One software (Figure 4.4 (b)). The protein profile indicates that most of the *P. kurroa* proteins have a molecular weight between 20 kDa to 97 kDa with the highest density of proteins located within the 20 kDa to 32 kDa range. In Figure 4.4 (a), five highly intense bands (band 3, 5, 6, 7 and 8) can be observed in the lane corresponding to the 15 ± 1 °C sample.

(a)



(b)

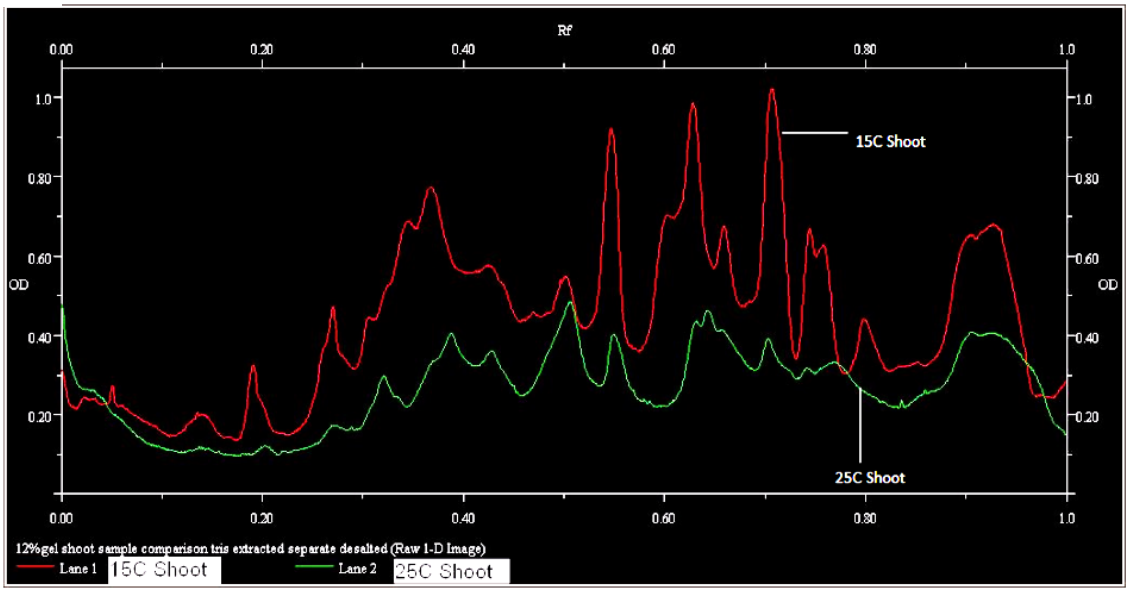


Figure 4.4 (a) SDS-PAGE profile of Tris-Cl extracted *P. kurroa* 15 ± 1 °C and 25 ± 1 °C shoot samples along with highlighted bands that were excised for mass spectrometric analysis; (b) Densitogram analysis of the gel using Bio-Rad Quantity One software

Table 4.1 Details of proteins identified in all the eight bands excised from SDS-PAGE gel of 15 ± 1°C maintained *P. kurroa* shoots vs 25 ± 1°C maintained *P. kurroa* shoots analyzed using MALDI-TOF MS and MASCOT database searching

Band No.	**Identified Protein**	**Molecular Mass (Da)**	**Isoelectric Point (pI)**	**Percent Sequence coverage**	**Protein Function**
1	ATP dependent Clp protease proteolytic subunit	24829	4.64	31%	• Cleaves peptides in various proteins in a process that requires ATP hydrolysis. • Has a chymotrypsin-like activity. • Plays a major role in the degradation of misfolded proteins.
2	ADP, ATP carrier protein	36837	9.85	20%	• Catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane.
3	Putative ROP family GTPase, ROP6	23561	9.39	50%	• Small GTPase mediated signal transduction and GTP Binding
4	30S ribosomal protein S3, chloroplastic	25159	9.85	28%	• rRNA Binding and Structural constituent of ribosomes
5	NAD(P)H-quinone oxidoreductase	27244	6.36	52%	• NADH shuttles electrons from NAD(P)H: plastoquinone, via FMN and iron-sulphur (Fe-S) centres, to quinones in the

	subunit K, chloroplastic				photosynthetic chain and possibly in a chloroplast respiratory chain. • Involved in cyclic electron transport around photosystem 1 hence, continuously supplies ATP for other pathways
6	Shikimate kinase	34192	7.66	23%	• Enzyme that catalyzes the formation of shikimate-3-phosphate which is the fifth step in biosynthesis of aromatic amino acid from chorismate • Shikimate kinase is involved in the biosynthesis of phenylpropanoids. • Phenylpropanoids are a diverse family of organic compounds that are synthesized by plants from the amino acid phenylalanine.
7	Ribulose bisphosphate carboxylase small chain, chloroplastic	19256	9.04	39%	• RuBisCO catalyzes the carboxylation of D-ribulose-1,5-bisphosphate, the primary event in carbon dioxide fixation. The reaction catalyzed by RubisCO is the rate-limiting step of Calvin cycle. • Feeds the MEP (non-mevalonate) pathway with glyceraldehyde-3-phosphate. This role might further serve two important functions a) Increased Chlorophyll synthesis b) Increased Monoterpene synthesis

8	Fructokinase (Fragment).	17994	5.61	50%	• Fructose is phosphorylated to fructose-6-phosphate by fructokinase and is used as a substrate for respiration and biosynthesis of starch and the other complex carbohydrates. • Suppressing the synthesis of fructokinase results in growth inhibition of stems and roots and reduction of flower and fruit number.

b) 2-D gel electrophoresis analysis

Biological replicates prepared by pooling protein extracted from a) shoots of three 15 ± 1 °C *P. kurroa* and b) shoots of three 25 ± 1 °C *P. kurroa* were analyzed using 2-D gel electrophoresis. Gels obtained from pooled extracts from each set of shoot samples were found to be highly reproducible. An average of 303 valid spots were detected in 15 ± 1 °C shoot samples as compared to an average of 219 spots in 25 ± 1 °C shoot samples (Figure 4.5).

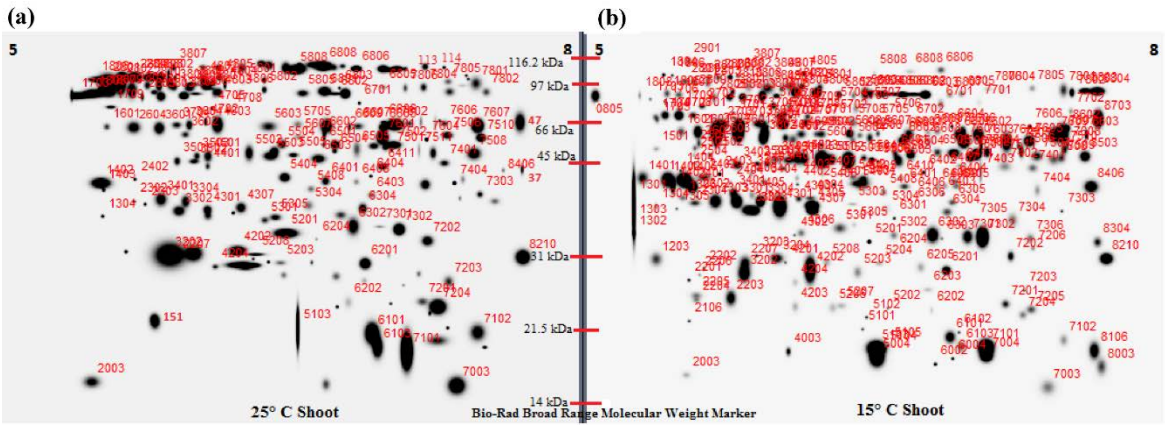


Figure 4.5 2-D gel electrophoresis profile of *P. kurroa* shoot samples grown under differential conditions of metabolite accumulation **(a)** metabolite non-accumulation condition (25 ± 1 °C) and **(b)** metabolite accumulation condition (15 ± 1 °C)

A total of 37 uniquely expressed, 33 up-regulated, 25 down-regulated proteins were detected in 15 ± 1 °C shoots. Interestingly, 5 spots were found to be missing from the protein map of 15 ± 1 °C shoots.

Identification and functional classification of differentially expressed proteins

Identification of differentially expressed proteins is imperative for a thorough understanding of the mechanisms responsible for the observed alterations in picroside-I content and phenotypic appearances amongst 15 ± 1 °C shoots and 25 ± 1 °C shoots. 2-D gel electrophoresis coupled with MALDI-TOF/TOF MS is a powerful tool for identifying differentially expressed proteins. All the 100 differentially expressed proteins identified using peptide mass fingerprint data derived from MALDI-TOF/TOF-MS whose relative expression levels in 15±1 °C shoots and 25±1 °C shoots are represented as a heatmap (Figure 4.6) were classified according to their biological functions into 7 distinct categories namely metabolism, transcription/translation, signal transduction, stress and defense,

transport, cell cycle and unknown. Table 4.2 shows an overview of protein expression pattern with respect to their functional classification.

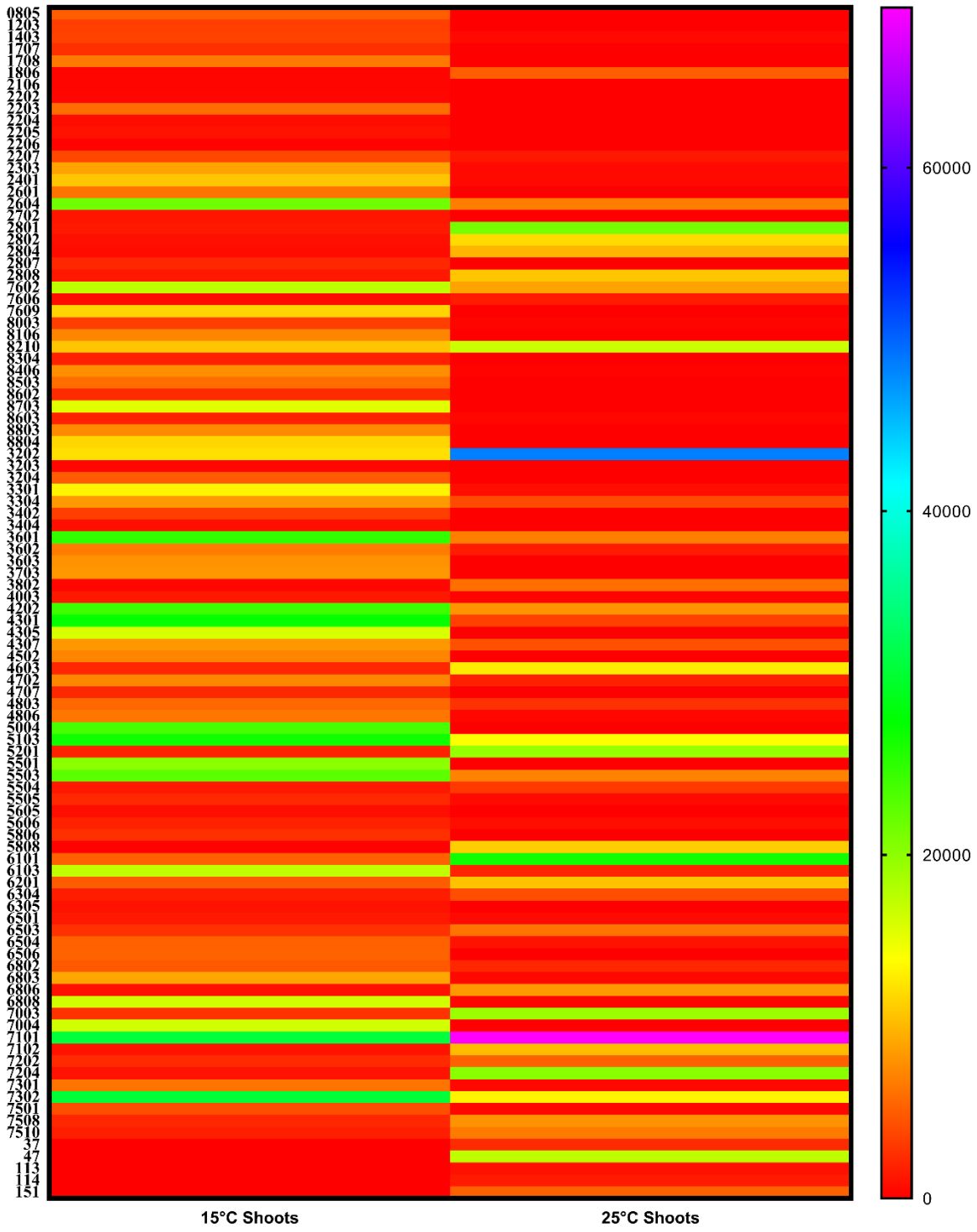


Figure 4.6 Heatmap demonstrating relative expression of 100 proteins in 15 ± 1 °C and 25 ± 1 °C *P. kurroa* shoots in response to low temperature stress and the ensuing enhanced picroside biosynthesis

Table 4.2 Protein expression pattern in terms of number of proteins with respect to their putative functional classification.

	Metabolism	Transcription /translation	Signaling	Stress and	Transport	Cell Cycle	Unknown
Unique expression in 15 ± 1 °C	20	3	5	4	2	0	3
Expression absent in 15 ± 1 °C	1	4	0	0	0	0	0
Up regulated in 15 ± 1 °C	22	1	3	2	2	0	3
Down regulated in 15 ± 1 °C	4	6	6	0	0	1	8

The largest group of proteins was metabolism-associated proteins (47%) followed by transcription/translation-associated proteins (14%), proteins with unknown biological functions (14%), signaling associated proteins (14%), stress and defense-associated proteins (8%), transporters (4%) and cell cycle associated proteins (1%) (Figure 4.7).

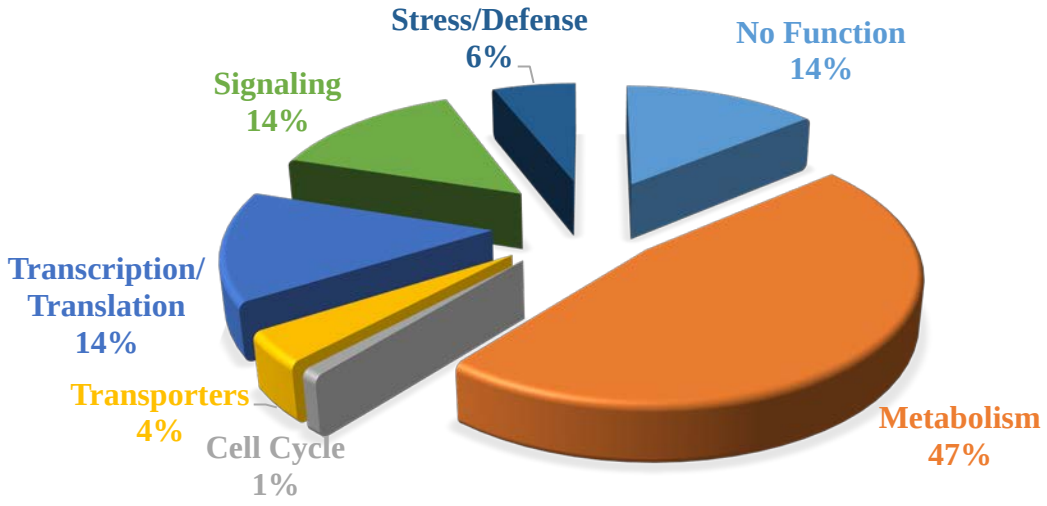
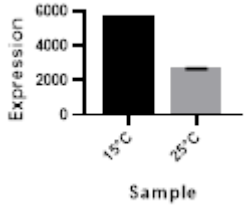
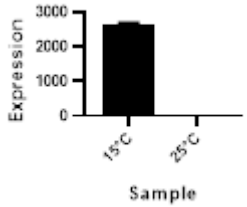
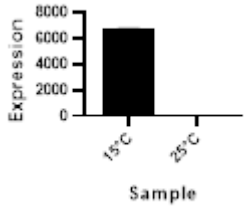
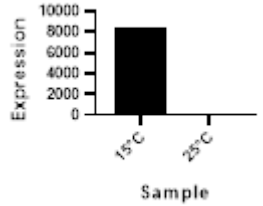
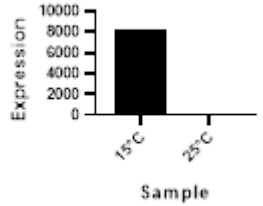
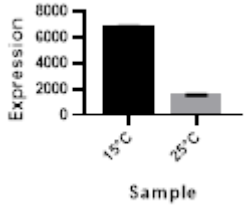


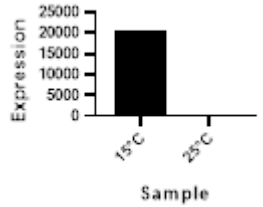
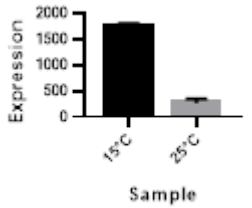
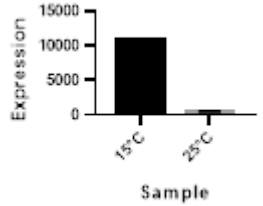
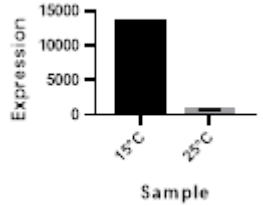
Figure 4.7 Putative functional classification of differentially expressed proteins in *P. kurroa*. Based on the biological function, the differentially expressed proteins were grouped into 7 distinct categories

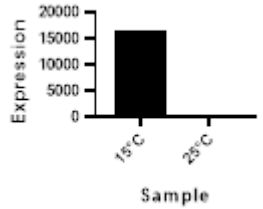
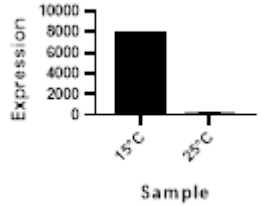
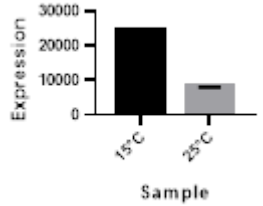
Differentially expressed proteins involved in enhanced picroside-I content, shoot biomass and other plant processes identified using MALDI-TOF MS followed by database searching using MASCOT search engine have been listed in Table 4.3 along with other details like molecular weight, pI, sequence coverage, score, mass values matched, e-value and expression levels in respective tissues.

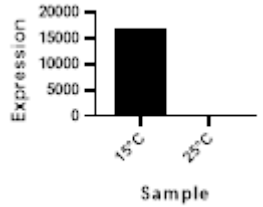
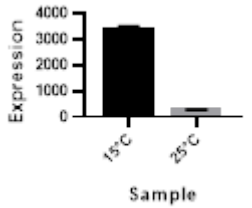
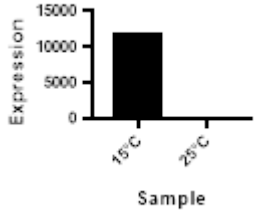
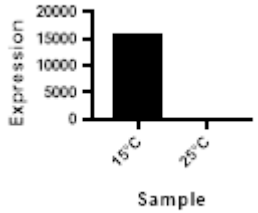
Table 4.3 Differentially expressed proteins involved in enhanced picroside-I content, shoot biomass and other plant processes identified using MALDI-TOF MS followed by database searching using MASCOT search engine

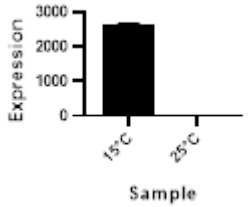
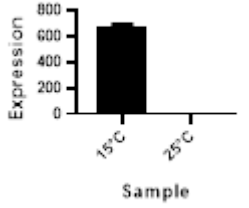
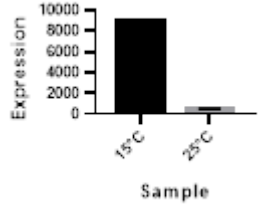
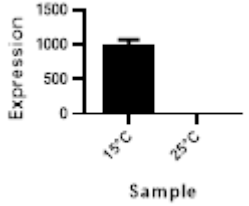
Spot No.	**Protein Name**	**ID**	**% Seq coverage**	**Mol. Wt.**	**pI**	**Score**	**Expect**	**Matches**	**Average expression level in 15 ± 1 °C and 25 ± 1 °C shoots (n=3, for each sample)**
4803	Phenylalanine ammonia-lyase 2 [*Arabidopsis thaliana*]	GI: 21264489	46	78381	6.03	74	0.04	14	
1707	Anthocyanin-5-aromatic acyltransferase [*Gentiana triflora*]	GI: 38503267	37	53162	5.57	73	0.052	15	

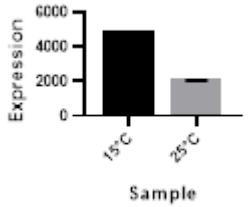
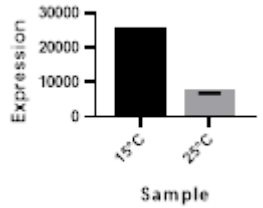
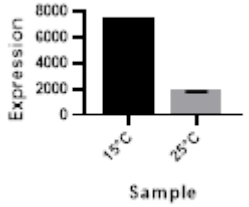
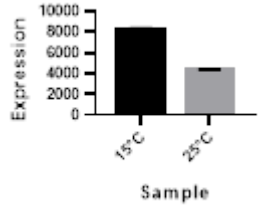
1708	Phospho-2-dehydro-3-deoxyheptonate aldolase 2, chloroplastic [*Solanum lycopersicum*]	GI: 584778	28	60299	7.43	74	0.04	14	
3703	ABC transporter [*Selaginella moellendorffii*]	GI: 302771345	40	76262	9.74	72	0.056	10	
3603	Cytochrome P450 monooxygenase, partial [*Stevia rebaudiana*]	GI: 218764507	31	54526	8.81	74	0.039	11	
3602	Isocitrate dehydrogenase [NADP], chloroplastic/mitochondrial-like [*Gossypium hirsutum*]	GI: 1029076682	36	55155	8.47	74	0.045	18	

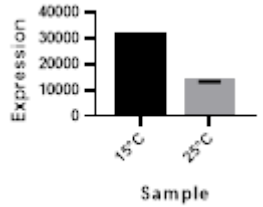
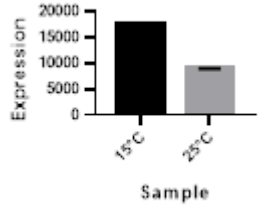
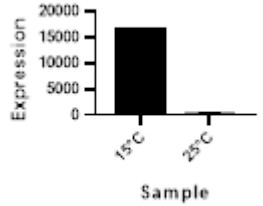
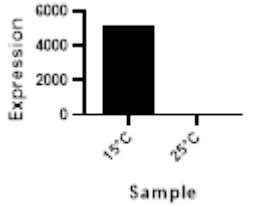
5501	Malate dehydrogenase [NADP], chloroplastic [*Pisum sativum*]	GI: 462579	35	48804	6.34	73	0.056	16	
8603	Aldehyde dehydrogenase family 2-member B7, mitochondrial like isoform X2 (*Cicer arietinum*)	GI: 502152285	17	47022	6.76	80	0.041	21	
2401	Probable fructokinase-6, chloroplastic [*Cucumis melo*]	GI: 659114466	26	41129	5.70	76	0.027	9	
3301	Heterodimeric geranylgeranyl pyrophosphate synthase large subunit 1,	GI: 950948561	30	34037	6.47	87	0.0078	24	

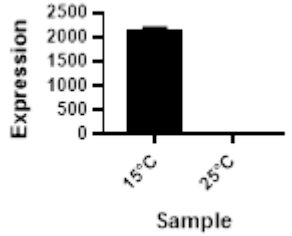
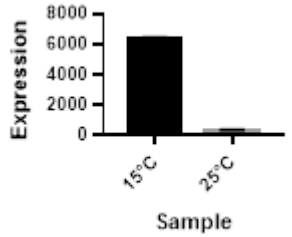
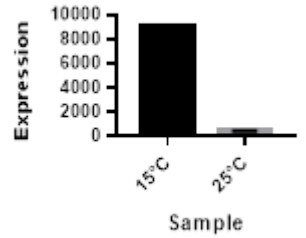
	chloroplastic-like [*Vigna radiata* var. radiata]								
4305	Glycosyltransferase [*Phaseolus vulgaris*]	GI: 543176396	26	56306	8.19	85	0.0038	11	
8406	Caffeic acid 3-O-methyltransferase-like [*Tarenaya hassleriana*]	GI: 729339747	19	43042	5.51	72	0.051	43	
4202	Fructose-1,6-bisphosphatase, cytosolic [*Daucus carota* subsp. sativus]	GI: 040891415	33	37117	5.42	76	0.021	19	

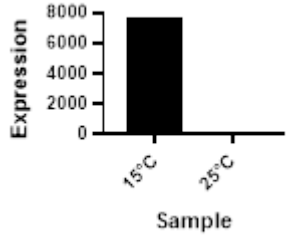
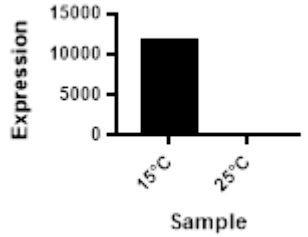
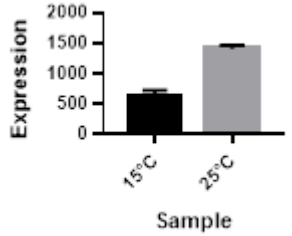
7004	Glyceraldehyde 3-phosphate dehydrogenase, partial [*Warneckea mangrovensis*]	GI: 62631804	87	13018	6.81	70	0.068	22	
8003	ABC transporter I family member 6, chloroplastic-like [*Nicotiana tomentosiformis*]	GI: 697095706	55	20073	6.90	80	0.0045	11	
7609	Type 2 1-hydroxy-2-methyl-2-(E)-butenyl-4-phosphate reductase [*Pinus taeda*]	GI: 126697262	31	55196	6.11	77	0.019	12	
8703	Malate synthase [*Coccomyxa subellipsoidea* C-169]	GI: 545360945	38	60578	8.37	77	0.02	26	

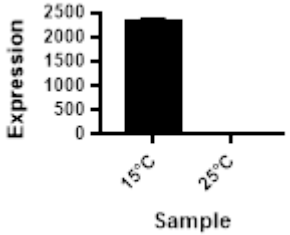
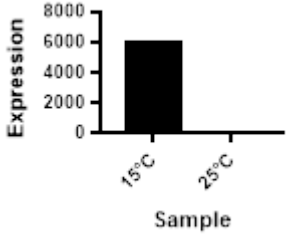
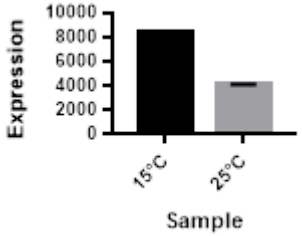
5806	ABC transporter B family member 4-like isoform X1 [*Eucalyptus grandis*]	GI: 702489398	20	138991	7.90	76	0.028	13	
5605	Geraniol 8-hydroxylase-like [*Solanum tuberosum*]	GI: 565342160	25	57727	8.09	75	0.035	11	
2303	3-dehydroquinate synthase, chloroplastic [*Solanum pennellii*]	GI: 970009841	31	48463	8.35	87	0.0082	20	
2205	Ribulose bisphosphate carboxylase small chain, chloroplastic-like [*Jatropha curcas*]	GI: 802681287	39	17648	8.90	75	0.039	10	

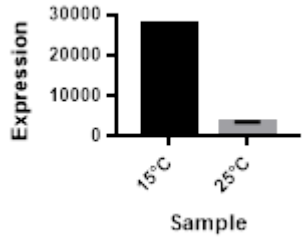
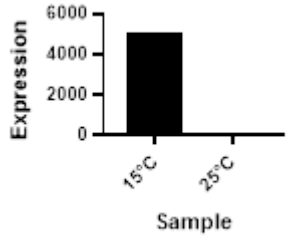
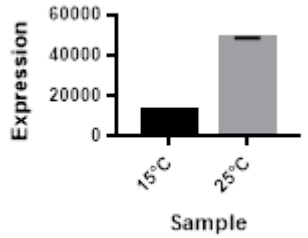
6802	ABC transporter B family member 1 [*Amborella trichopoda*]	GI: 586691120	17	146224	8.43	75	0.041	19	
3601	1-deoxy-D-xylulose-5-phosphate synthase [*Andrographis paniculata*]	GI: 40317612	20	63131	6.67	77	0.015	9	
4702	ATP-dependent 6-phosphofructokinase 5, chloroplastic [*Tarenaya hassleriana*]	GI: 729468321	28	57859	8.54	70	0.067	11	
4307	Glyceraldehyde-3-phosphate dehydrogenase [*Physcomitrella patens* subsp. Patens]	GI: 462139	37	36910	6.19	79	0.012	14	

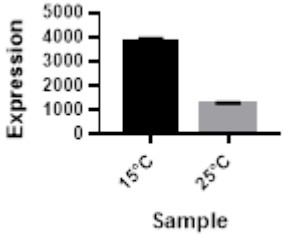
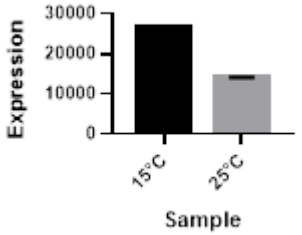
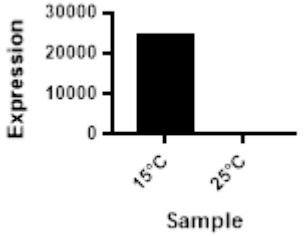
7302	3-deoxy-7-phosphoheptulonate synthase [*Lactuca Sativa*]	GI:73912416	21	30225	7.22	70	0.061	10	
7602	UDP-glycosyltransferase 73B3-like [*Glycine max*]	GI: 356499777	38	54267	5.78	73	0.046	11	
6808	Pyruvate, phosphate dikinase 2 [*Vigna angularis*]	GI: 1044540114	33	104648	5.75	79	0.01	32	
0805	Sucrose-phosphate synthase 4 [*Vigna angularis*]	GI: 1044576921	18	119681	5.99	81	0.0085	14	

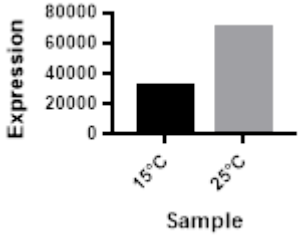
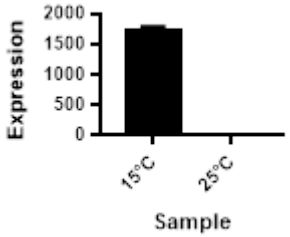
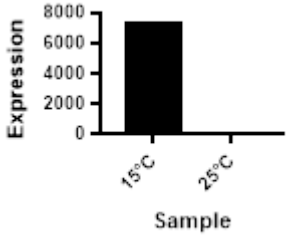
2807	Hypothetical protein osj_32941 [*Oryza sativa* Japonica Group]	GI: 222615508	30	86752	6.61	74	0.03	15	
4806	9-cis-epoxycarotenoid dioxygenase NCED6, chloroplastic [*Nicotiana sylvestris*]	GI: 698519185	27	66553	6.67	72	0.058	12	
6803	Transposon protein, putative, CACTA, En/Spm sub-class [*Oryza sativa* Japonica Group]	GI: 77551246	32	76653	8.64	72	0.063	12	

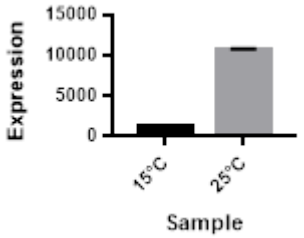
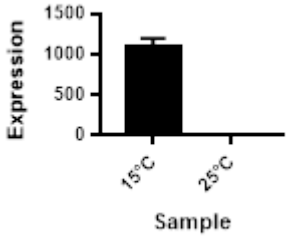
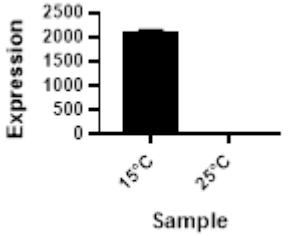
8803	Cactin-like isoform X1 [*Pyrus x bretschneideri*]	GI: 694401147	48	79211	6.21	74	0.045	27	
8804	Serine/threonine-protein kinase DDB_G0276461 isoform X1 [*Vitis vinifera*]	GI: 731415577	56	79526	7.92	78	0.019	18	
7606	Inositol 1,3,4-trisphosphate 5/6-kinase 4 isoform X2 [*Cucumis melo*]	GI: 659110223	12	60819	5.52	78	0.015	6	

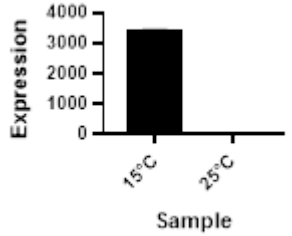
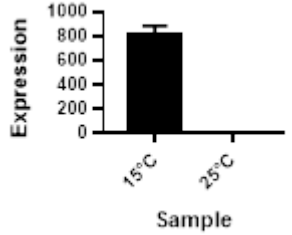
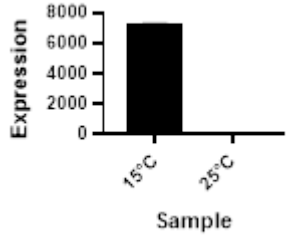
8602	Hypothetical protein KFL_002190110 [*Klebsormidium flaccidum*]	GI: 971513101	33	57239	9.86	74	0.039	27	
8503	Molecular chaperone (dnaj superfamily) (ISS) [*Ostreococcus tauri*]	GI: 308804519	35	48568	7.7	74	0.03	15	
3304	Cinnamyl alcohol dehydrogenase 6 [*Glycine soja*]	GI: 734418163	31	39767	6.63	70	0.1	24	

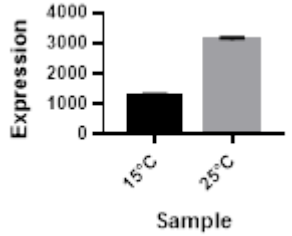
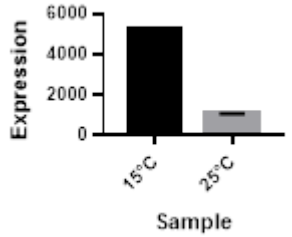
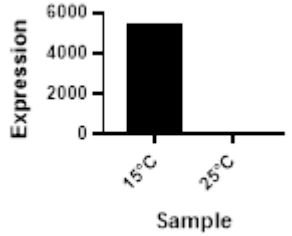
4301	LOV domain-containing protein [*Nicotiana sylvestris*]	GI: 1001015069	57	45030	8.49	71	0.066	20	
3204	histidine-containing phosphotransfer protein 1-like isoform X1 [*Citrus sinensis*]	GI: 985438883	28	29359	6.49	73	0.048	24	
3202	Zinc-finger homeodomain protein 3 [*Erythranthe guttata*]	GI: 848879735	43	24645	8.93	75	0.034	23	

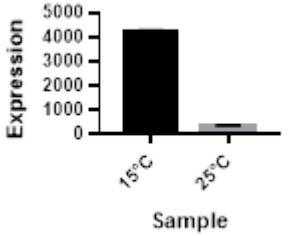
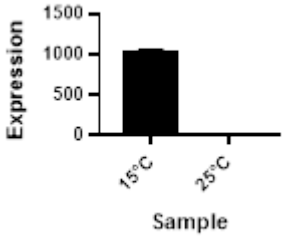
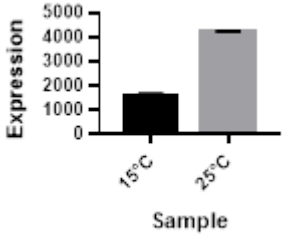
2207	Protease Do-like 14 [*Beta vulgaris* subsp. Vulgaris]	GI: 731356780	52	27407	9.47	73	0.047	16	
5103	DNA polymerase epsilon subunit 3-like [*Brachypodium distachyon*]	GI: 721682580	44	19141	4.93	76	0.026	13	
5004	Protein COLD-REGULATED 15B, chloroplastic [*Brassica napus*]	gi|923673518	47	20416	9.05	77	0.01	15	

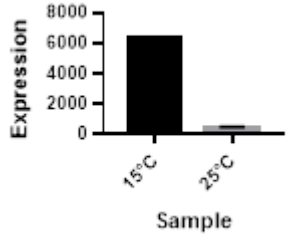
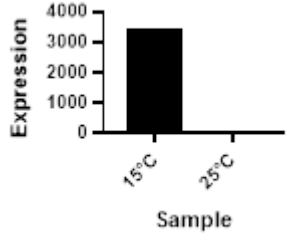
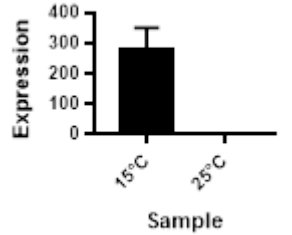
7101	Peptidase S24/S26A/S26B/S26C family protein [*Klebsormidium flaccidum*]	gi|971513224	49	20465	8.86	70	0.07	8	
8304	Cytochrome b-c1 complex subunit Rieske-4, mitochondrial-like [*Prunus mume*]	GI: 645250484	23	30293	8.54	73	0.042	9	
8106	Two-component response regulator ARR15-like isoform X2 [*Populus euphratica*]	GI: 743820091	62	19952	9.13	74	0.03	10	

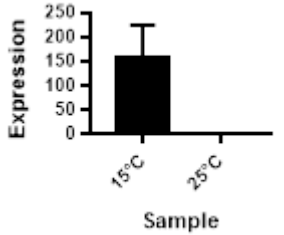
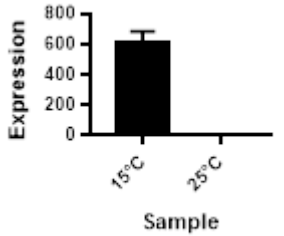
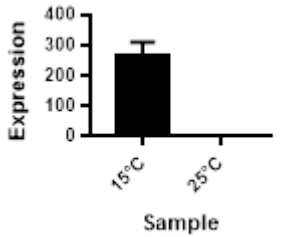
2808	ADP-ribosylation factor gtpase-activating protein AGD3-like isoform X2 [*Daucus carota* subsp. Sativus]	GI: 1040901138	29	91679	8.32	74	0.037	10	
2702	Cingulin-like protein 1 [*Arachis ipaensis*]	GI: 1021524204	22	66976	5.16	70	0.06	10	
4707	Chaperonin 60 subunit beta 2, chloroplastic [*Brassica rapa*]	GI: 685288707	25	63079	5.76	80	0.0037	14	

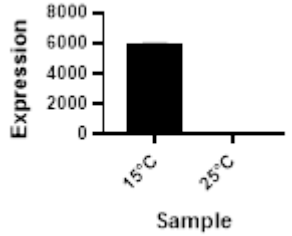
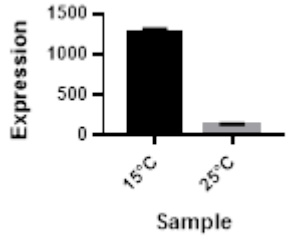
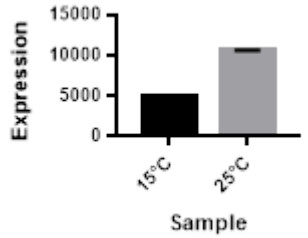
3402	3-hydroxyisobutyryl-coa hydrolase 1-like [*Solanum pennellii*]	GI: 969996575	32	42909	7.11	76	0.026	7	
3404	Hypothetical protein CARUB_v10023284mg [*Capsella rubella*]	GI: 565471334	27	46649	8.41	73	0.045	7	
4502	Radical SAM superfamily protein [*Theobroma cacao*]	GI: 590627706	30	42110	7.06	76	0.011	12	

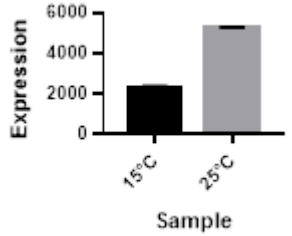
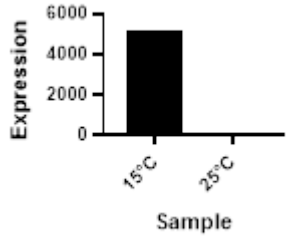
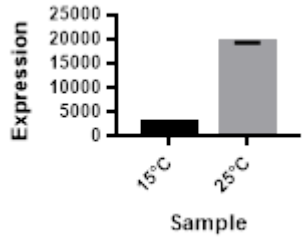
5504	Protein phosphatase 2C 76 [*Camelina sativa*]	GI: 727537857	44	45852	5.03	73	0.044	18	
6504	Aspartate carbamoyltransferase 1, chloroplastic [*Nicotiana sylvestris*]	GI: 698445307	30	45639	6.43	74	0.026	17	
6506	Jasmonate O-methyltransferase-like [*Malus domestica*]	GI: 658038176	43	41081	5.36	71	0.067	8	

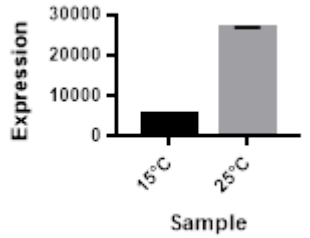
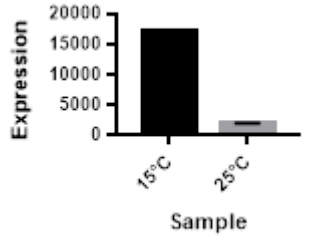
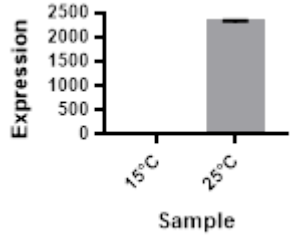
7501	Phosphatidylserine decarboxylase proenzyme 2-like isoform X2 [*Nicotiana tomentosiformis*]	GI: 697136430	34	46762	8.25	71	0.065	8	
6305	NAD-dependent ADP-ribosyltransferase sirtuin-4, partial [*Cajanus cajan*]	GI: 1012336149	40	40351	8.93	74	0.04	16	
6304	F-box/kelch-repeat protein At1g20940 [*Camelina sativa*]	GI: 727608557	29	45140	8.42	73	0.047	11	

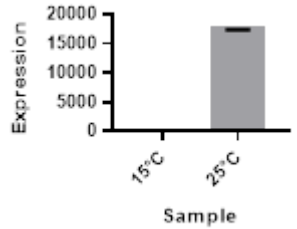
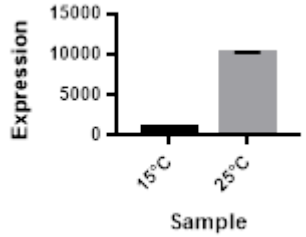
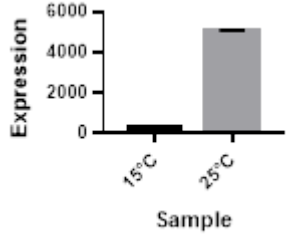
7301	7-methylguanosine phosphate-specific 5'-nucleotidase A [*Ziziphus jujuba*]	GI: 1009171569	26	40129	5.87	73	0.049	15	
1203	Strictosidine synthase [*Aegilops tauschii*]	GI: 475356850	38	23146	9.30	76	0.028	9	
2202	Anthocyanidin 5,3-O-glucosyltransferase [*Aegilops tauschii*]	GI: 475532016	45	34663	7.11	73	0.44	16	

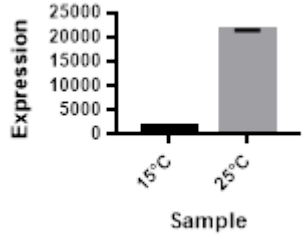
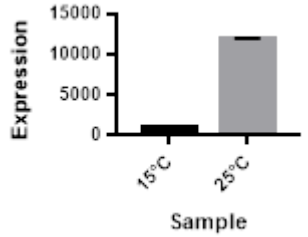
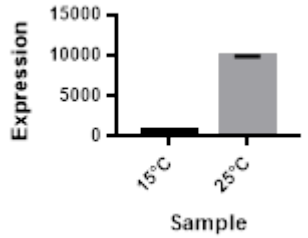
2206	caffeoyl-CoA O-methyltransferase At4g26220 [*Solanum lycopersicum*]	GI: 460389351	57	24940	5.61	77	0.018	12	
2204	Nudix hydrolase 16, mitochondrial-like [*Dorcoceras hygrometricum*]	GI: 1024062979	56	20911	5.62	70	0.07	10	
2106	Ribosomal protein small subunit 4, partial (chloroplast) [*Gaga arizonica*]	GI: 118724455	33	21246	10.16	90	0.0038	15	

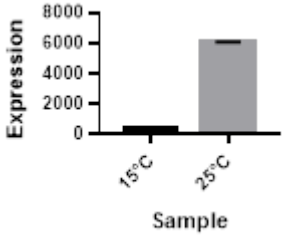
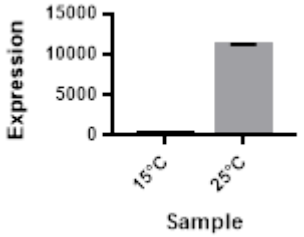
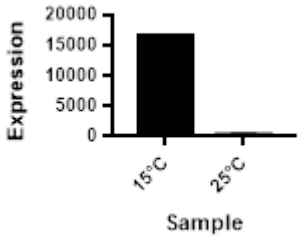
2203	Late embryogenesis abundant protein 47-like [*Gossypium hirsutum*]	GI: 1029060073	53	26263	6.35	72	0.053	15	
4003	Probable caffeoyl-CoA O-methyltransferase At4g26220 [*Solanum lycopersicum*]	GI: 460389351	57	24940	5.61	80	0.01	13	
6201	Matrixin family protein, putative [*Theobroma cacao*]	GI: 590566097	38	32640	5.86	72	0.05	12	

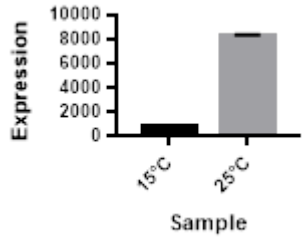
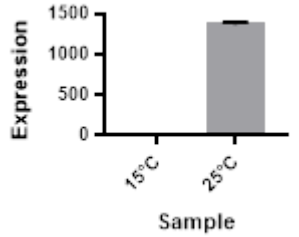
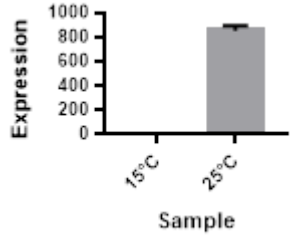
7202	Uncharacterized protein LOC104598296 isoform X2 [*Nelumbo nucifera*]	GI: 720008337	17	31383	8.45	76	0.018	8	
0805	Probable sucrose-phosphate synthase 4 [*Vigna angularis*]	GI: 1044576921	18	119681	5.99	81	0.0085	14	
7003	CDPK-related protein kinase-like [*Nicotiana tomentosiformis*]	GI: 697098786	80	17943	5.87	81	0.0066	7	

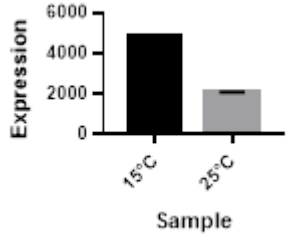
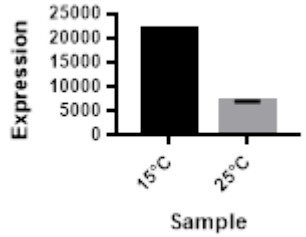
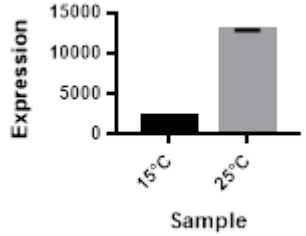
6101	Ribosomal protein S11 (chloroplast) [*Goodyera fumata*]	GI: 810932605	90	15077	12.01	70	0.073	10	
6103	Nudix hydrolase 16, mitochondrial-like [*Dorcoceras hygrometricum*]	GI: 1024062979	60	20911	5.62	74	0.037	10	
37	Protein phosphatase [*Populus trichocarpa*]	GI: 566210556	52	40412	5.17	70	0.063	38	

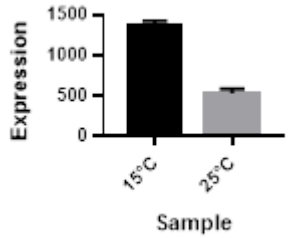
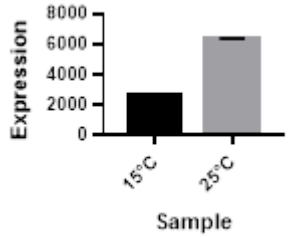
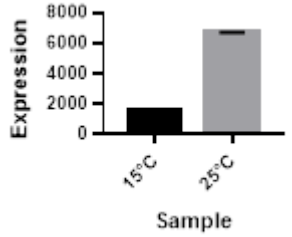
47	Glutamyl-tRNA synthetase, cytoplasmic [*Triticum urartu*]	GI: 474262559	33	77816	7.23	81	0.008	19	
7102	Hypothetical protein KFL_003000010 [*Klebsormidium flaccidum*]	GI: 971511582	39	19264	4.87	74	0.041	8	
1806	Predicted protein [*Physcomitrella patens*]	GI: 168010021	10	97717	6.71	77	0.025	26	

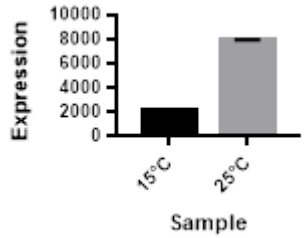
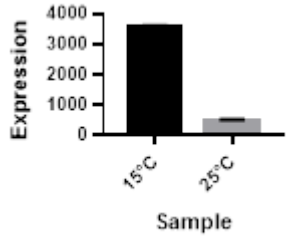
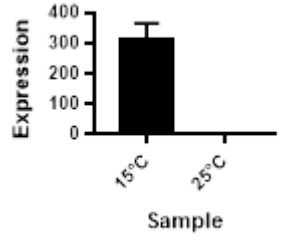
2801	Hypothetical protein JCGZ_00460 [*Jatropha curcas*]	GI: 643707975	21	98302	6.04	83	0.0057	25	
2802	Hypothetical protein BRADI_1g06907 [*Brachypodium distachyon*]	KQK12934.1	25	97095	7.01	77	0.031	25	
2804	RNA polymerase beta chain (chloroplast) [*Gnetum ula*]	GI: 966203134	18	127653	9.10	70	0.065	11	

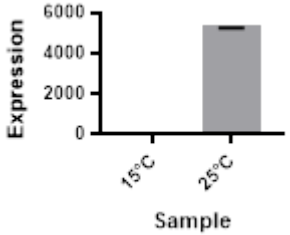
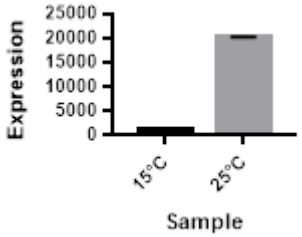
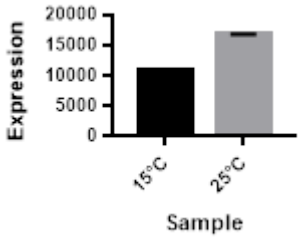
3802	TIR-NBS-LRR class disease resistance protein [*Arabidopsis thaliana*]	GI: 15235064	18	134190	7.48	70	0.071	22	
5808	Bnac04g54780d [*Brassica napus*]	GI: 674868946	31	93526	11.67	77	0.017	30	
6808	Pyruvate, phosphate dikinase 2 [*Vigna angularis*]	GI: 1044540114	33	104648	5.75	79	0.01	32	

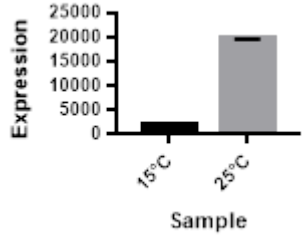
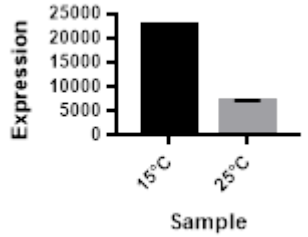
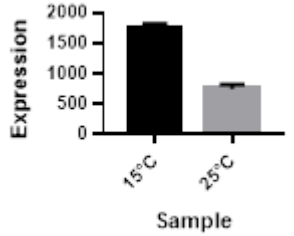
6806	Glutamine--tRNA ligase [*Tarenaya hassleriana*]	GI: 729386066	24	91894	6.23	70	0.08	13	
113	Argonaute protein group [*Theobroma cacao*]	GI: 590621822	19	101080	9.25	76	0.02	10	
114	U3 small nucleolar RNA-associated protein 21 homolog isoform X1 [*Cicer arietinum*]	GI: 502164326	16	103233	6.40	70	0.077	17	

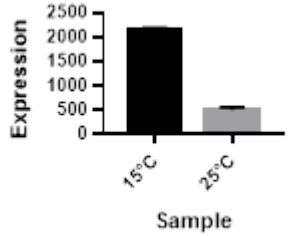
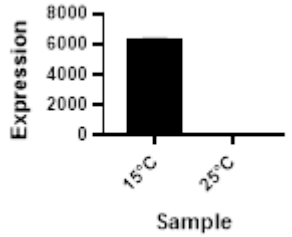
6802	ABC transporter B family member 1 [*Amborella trichopoda*]	GI: 586691120	17	146224	8.43	75	0.041	19	
2604	Hypothetical protein POPTR_0005s23880g [*Populus trichocarpa*]	GI: 224082718	23	76881	7.81	72	0.051	12	
4603	Serine/threonine-protein kinase Nek2 [*Setaria italica*]	GI: 514754751	11	66752	9.50	70	0.073	11	

6501	Disease resistance protein RGA1 [*Eucalyptus grandis]*	GI: 702359424	54	43320	5.94	70	0.082	17	
6503	Predicted protein [*Bathycoccus prasinos*]	GI: 612391813	28	51809	9.68	75	0.048	13	
7510	Nuclear RNA export factor SDE5 [*Malus domestica*]	GI: 658047342	28	52185	5.91	74	0.031	11	

7508	N-acetyltransferase HLS1 [*Gossypium arboreum]*	GI: 1050644131	53	43752	9.49	82	0.0075	22	
1403	Glycogen synthase kinase-3 homolog msk-3 [*Prunus mume*]	GI: 645243071	28	46679	8.70	75	0.03	6	
3203	NAC domain-containing protein 21/22 [*Zea mays*]	GI: 226530985	34	34749	7.12	81	0.009	43	

151	Transcription factor bhlh30-like [*Phoenix dactylifera*]	GI: 672190344	58	21786	10.07	71	0.085	12	
7204	Hypothetical protein MIMGU_mgv1a021377mg [*Erythranthe guttata*]	GI: 604341195	52	15187	8.56	76	0.024	6	
8210	Cyclin-dependent protein kinase inhibitor SMR3-like isoform X1 [*Gossypium raimondii*]	GI: 823175341	35	16802	5.10	81	0.0095	12	

5201	Translation elongation factor-like protein, partial [*Parachlorella kessleri*]	GI: 224593225	22	34932	7.88	76	0.028	8	
5503	Hypothetical protein EUTSA_v10020781mg [*Eutrema salsugineum*]	GI: 567199862	46	49203	8.81	72	0.05	17	
5606	Serine/threonine-protein kinase PEPKR2 [*Vitis vinifera*]	GI: 359492476	13	52045	9.05	93	0.0005 7	9	

5505	Alcohol dehydrogenase [*Fourraea alpina*]	GI: 6684367	23	41880	5.84	82	0.0092	12	
2601	Ankyrin repeat domain-containing protein 65 [*Amborella trichopoda*]	GI: 586642473	49	56374	8.45	70	0.069	21	

Proteins involved in metabolism

In case of 15 ± 1 °C shoot samples, 20 proteins (1707, 1708, 3603, 7004, 7609, 8703, 5605, 3402, 4502, 6506, 1203, 2202, 2206, 2205, 0805, 2204, 6305, 4305, 5501 and 8304) were uniquely expressed and 22 proteins (4803, 4806, 3602, 8603, 2401, 3301, 3304, 8406, 4202, 6504, 7501, 2303, 7301, 4003, 3601, 4702, 4307, 5505, 7302, 6103, 7602, and 6808) were up-regulated. 4 proteins (6304, 6201, 7003 and 7508) were found to be down-regulated while 1 protein (37) was absent in 15 ± 1 °C shoot samples.

Proteins involved in transcription/translation

Three proteins (2106, 3203 and 8803) were uniquely expressed, and 1 protein (5103) was up-regulated in 15 ± 1 °C shoot samples while 4 proteins (47, 113, 114 and 151) were absent, and 6 proteins (3202, 6101, 2804, 6806, 7510 and 5201) were observed to be down-regulated in 15 ± 1 °C shoots.

Proteins involved in signalling

Five proteins (3204, 8106, 2702, 2601 and 8804) were uniquely expressed, 3 proteins (4301, 1403 and 5606) were up-regulated in 15 ± 1 °C shoots, and 6 proteins (7606, 7101, 2808, 5504, 3802 and 4603) were downregulated in 15 ± 1 °C shoot samples.

Proteins involved in stress and defense

Four proteins (8503, 4707, 2203 and 5004) were uniquely expressed, and 2 proteins (2207 and 6501) were up-regulated in 15 ± 1 °C shoots.

Proteins involved in transport

In 15 ± 1 °C shoots, 2 proteins (3703 and 5806) were uniquely expressed, and 2 proteins (6802 and 8003) were observed to be up-regulated.

Proteins involved in cell cycle

One protein (8210) was observed to be down-regulated in 15 ± 1 °C shoots.

Proteins with unknown function

Some proteins whose biological functions are still not known were also identified. Amongst them 3 proteins (2807, 8602 and 3404) were uniquely expressed, 3 proteins (2604, 5503 and 6803) were up-regulated, and 8 proteins (7202, 7102, 1806, 2801, 2802, 5808, 6503 and 7204) were down-regulated in 15 ± 1 °C shoots.

4.3 Western Blot analysis

Western blot analysis was used to ascertain that the expression of glyceraldehyde-3-phosphate dehydrogenase, was evidently being increased in the tissues associated with picroside biosynthesis. The analysis revealed that the expression of glyceraldehyde-3-phosphate dehydrogenase was enhanced in 15 ± 1 °C shoots as compared to 25 ± 1 °C shoots which is in accordance with the observation in differential protein expression.

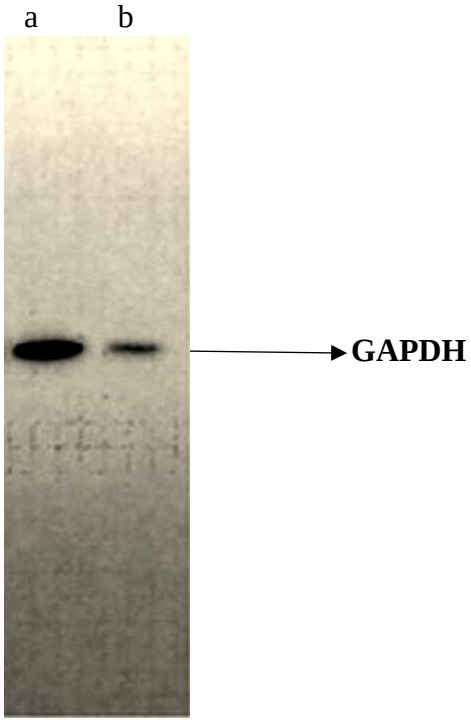


Figure 4.8 Reaction profiles in Western blot. Lane (a) 15 ± 1 °C Shoot, (b) 25 ± 1 °C Shoot

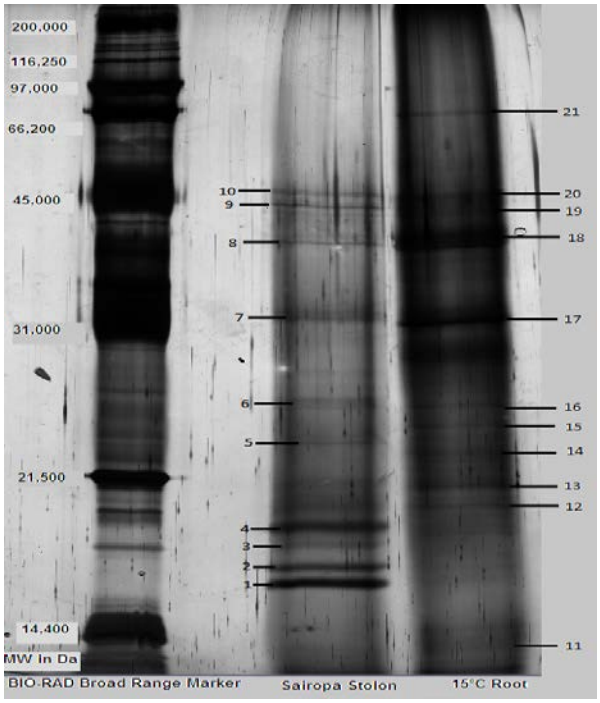
4.4 Differential protein expression studies in *P. kurroa* to investigate the presence of proteins involved in the biosynthesis of picroside-I and picroside-II in stolons.

SDS-PAGE analysis

Differential protein expression studies revealeda total of 29 bands in Sairopa and 2 6 bands in 15 ± 1 °C root samples (Figure 4.9 (a)). Densitometry analysis (Figure 4.9 (b)) of the gel using Bio-Rad's Quantity One software identified a total of 21

differentially expressed proteins, out of which 10 proteins were differentially expressed in Sairopa stolons while 11 were differentially expressed in 15 ± 1 °C roots. Gel analysis showed that most of the proteins were concentrated between a molecular weight range of 15 kDa to 45 kDa.

(a)



(b)

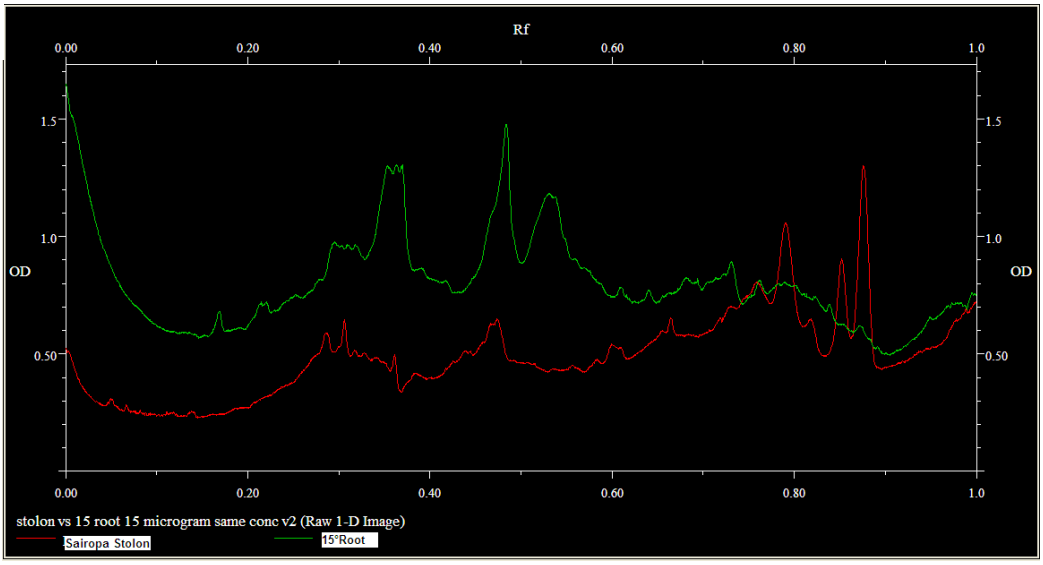


Figure 4.9 (a) SDS-PAGE profile of Sairopa stolon and 15 ± 1 °C roots with marked bands that were excised for MALDI-TOF MS analysis based on their differential expression (b) Densitogram analysis of SDS-PAGE gel using Bio-Rad's Quantity One software showing

the relative optical densities and relative front of various bands. The red line represents Sairopa stolon samples and the green line represents 15 ± 1 °C root samples.

Identification of differentially expressed proteins

Mass by charge ratios of 21 differentially expressed proteins obtained from MALDI-TOF/TOF MS was used to search the MASCOT database. Out of 21, 19 differentially expressed proteins were identified. Table 4.4 shows the differentially expressed proteins identified after MASCOT analysis. These proteins were involved in stress response, signaling pathways, metabolic pathway, transcription, and energy metabolism. The functional distribution of proteins is represented in Figure 4.10. The majority of proteins were found to be involved in stress response and metabolic pathways.

Table 4.4 Differentially expressed proteins identified using MALDI-TOF MS data and MASCOT search engine from stolons of *P. kurroa* obtained from Sairopa and roots of tissue cultured *P. kurroa* maintained at 15 ± 1°C

Band No.	Protein Identified	Accession number	M.W. (Da)	% Seq. Coverage	SCORE	Functions
1	Methionine sulfoxide reductase	XP_003594067	15,446	29	54	• Methionine sulfoxides can be reduced back to the methionines by a thioredoxin-dependent enzyme, peptide methionine sulfoxide reductase (Msr), providing cells with a mechanism to repair proteins damaged by reactive oxygen species rather than having them degraded and then re-synthesizing them *de novo*.
2	Ferredoxin-6	NP_001147617	15,334	14	60	• It uses iron-sulphur proteins as electron donors and NAD^+ or $NADP^+$ as electron acceptors. These function primarily in photosynthesis where they transfer electrons from photo reduced Photosystem I to ferredoxin NADP (+) reductase in which NADPH is produced for CO_2 assimilation.

3	40S ribosomal protein S13-1	Q69UI2	17,105	46	59	• Is involved in protein translation.
4	Peptidyl-prolyl cis-trans isomerase	P35627	18,320	27	55	• Peptidyl-prolyl cis-trans isomerase has been termed conformases or rotamases because they catalyze slow steps in the initial folding/rearrangement of proteins.
5	RAB GTPase B1C isoform 2	EOY29043	21,168	43	52	• Key players of vesicular transport. They act as molecular switches regulating the fusion of vesicles with target membranes through the conformational change between GTP- and GDP-bound forms.
6	DnaJ homolog subfamily C member 28-like	XP_004497574	22,904	40	54	• Play central roles in the heat stress. Act as co-chaperone along with DnaK in the DnaK-DnaJ system. DnaJ is able to act as a chaperone on its own, but its main role is as co-chaperone interacting with DnaK.
7	Glyceraldehyde-3-phosphate dehydrogenase	ABD37966	34,541	20	68	• Enzyme of the glycolytic pathway responsible for the conversion of glyceraldehyde 3-phosphate to D glycerate-1, 3,-bisphosphate. It

						also acts as a reversible metabolic switch under oxidative stress.
8	1-aminocyclopropane-1-carboxylate oxidase	XP_002532366	38,381	25	56	• Plays an important role in the regulation of ethylene formation in plants in response to various stress conditions.
10	1-acyl-sn-glycerol-3-phosphate acyltransferase 2-like	XP_004136588	43,818	17	61	• It is a key enzyme of phospholipid and triglyceride biosynthesis. Several lines of evidence suggest that AGPAT should play a key role in the synthesis of phospholipids/triglycerides required for permeability barrier homeostasis.
11	Photosystem I reaction centre subunit V, chloroplast precursor	XP_002949489	13,343	30	72	• Encodes subunit G of photosystem I, an 11 kDa membrane protein that plays an important role in electron transport between plastocyanin and PSI and is involved in the stability of the PSI complex. It also takes part in cyclic photophosphorylation resulting in ATP production without NADPH generation.

12	2-oxoglutarate ferrous-dependent oxygenase	ADY80557	20,760	27	52	• The 2-oxoglutarate (2-OG) and ferrous iron-dependent oxygenases are a superfamily of enzymes that catalyze a wide range of reactions including hydroxylations, desaturations, and oxidative ring closures. 2-OG oxygenases catalyze reactions in the post-translational modification of collagens and in the biosynthesis of both primary and secondary metabolites. They have also been identified as catalyzing steps in the biosynthesis of plant signaling molecules including ethylene and the gibberellins.
13	Predicted: ethylene-responsive transcription factor WIN1-like	XP_004152351	21,950	33	57	• Promotes cuticle formation by inducing the expression of enzymes involved in wax biosynthesis. Confers drought resistance. Acts as a transcriptional activator. Binds to the GCC-box pathogenesis-related promoter element.
14	UbiE/COQ5 methyltransferase	XP_003078783	24,603	36	66	• Involved in ubiquinone biosynthesis.

15	Putative cytochrome P450 superfamily protein	AFW88279	23,550	25	64	• Cytochrome P450 (CYP) enzymes are a superfamily of mono-oxygenases that are involved in the biosynthesis of several compounds, such as hormones, defensive compounds, and fatty acid conjugates.
16	NADH dehydrogenase subunit F	ABB93079	27,880	28	52	• Catalyses the transfer of electrons from NADH to coenzyme Q (CoQ). It is the "entry enzyme" of oxidative phosphorylation in the mitochondria.
17	Mitochondrial carrier family	XP_002502691	29,524	36	59	• Solute carriers in the inner mitochondrial membrane which connect the internal metabolism with that of the surrounding cell. It is known to catalyze the specific transport of various substrates, such as nucleotides, amino acids, dicarboxylates, cofactors, phosphate or H^+.
18	Adenylate isopentenyl transferase 8	Q9LJL4	37,583	23	67	• Involved in cytokinin biosynthesis. Catalyzes the transfer of an isopentenyl group from dimethylallyl diphosphate (DMAPP) to ATP and ADP.

20	Chaperone protein DnaJ-like	XP_002274349	48,696	13	55	• Members of the DnaJ-like protein family act as chaperones through direct interaction with different Hsp70 acting in protein pairs that appear to be specifically adapted to each other.
21	3-ketoacyl-CoA synthase 11-like isoform 1	XP_004251584	57,764	12	60	• Contributes to cuticular wax and suberin biosynthesis. Involved in both decarbonylation and acyl-reduction wax synthesis pathways.

Proteins involved in: a) Stress response include methionine sulfoxide reductase, peptidyl-prolyl cis-trans isomerase, DNAJ homolog subfamily C, glyceraldehyde-3-phosphate dehydrogenase, 1-aminocyclopropane-1-carboxylate oxidase, and chaperone protein DnaJ; b) Signaling pathways include Rab GTPase; c) Metabolism related activities include glyceraldehyde-3-phosphate dehydrogenase, 1-acyl-sn-glycerol-3-phosphate acyltransferase 2, 2-oxoglutarate ferrous-dependent oxygenase, UbiE/COQ5 methyltransferase, putative cytochrome P450 superfamily protein, adenylate isopentenyl transferase and 3-ketoacyl-CoA synthase 11; d) Transcription and translation include predicted ethylene-responsive transcription factor WIN1 and 40S ribosomal protein & e) Energy metabolism include ferredoxin, photosystem 1 reaction centre subunit V, NADH dehydrogenase subunit F and mitochondrial carrier protein.

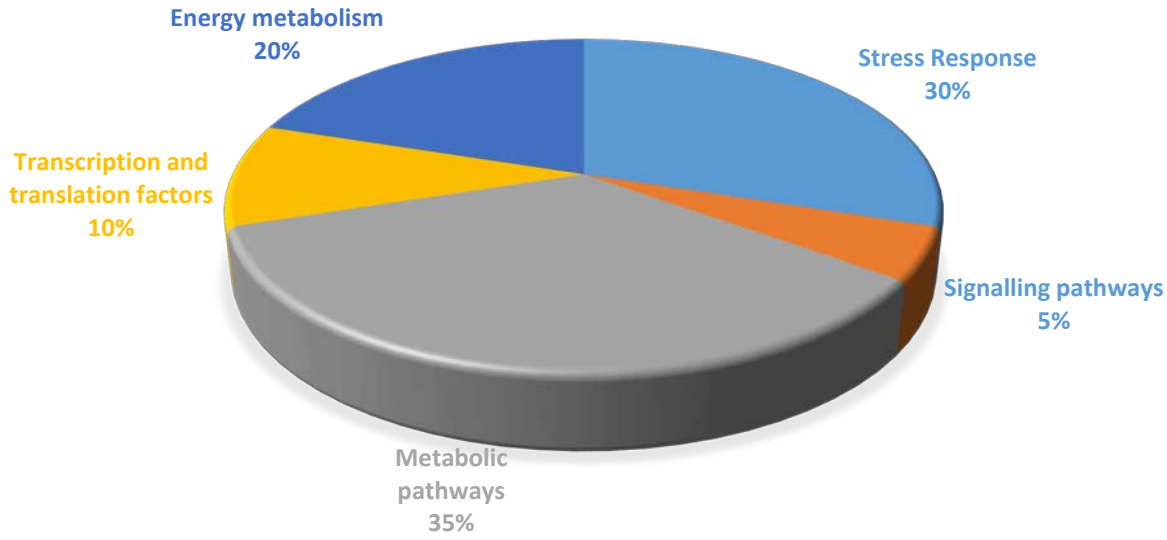


Figure 4.10 Putative functional classification of differentially expressed proteins identified in stolons of *P. kurroa* obtained from Sairopa and roots of tissue cultured *P. kurroa* maintained at 15°C

CHAPTER 5
DISCUSSION

5.1 Optimization of protein extraction protocol

In this study, we analyzed various protein extraction protocols for sample preparation and have carried out differential protein expression studies to identify imperative enzymes. Sample preparation is one of the most critical steps since a proteome consists of many proteins which have different localization, abundance, sub-localization and chemical properties [265]. As proteins differ among themselves in their physical and chemical properties different extraction protocols may favour proteins from different materials [266]. Therefore, an attempt was made to identify the extraction method which would give maximum protein yield. For shoots and roots, the highest protein concentration was obtained by Tris-Cl extraction method followed by phosphate buffer extraction method and then by TCA-Acetone extraction method. In case of stolons highest protein yield was obtained by TCA/Acetone method, followed by phosphate buffer and then Tris-Cl extraction method. As expected the lowest protein concentration was obtained using water extraction method which did not contain any reagent to increase protein solubilization.

5.2 Differential protein expression studies in *P. kurroa* to identify proteins involved in the biosynthesis of picroside-I and increased shoot biomass

a) SDS-PAGE

Analysis of metabolites, proteins and transcriptional profiling under two physiological states (for example metabolites accumulating versus non-accumulating) can provide an approach for identifying proteins involved in the biosynthesis of metabolites and other observed differences [85]. Therefore, for the present study, 15 ± 1 °C and 25 ± 1 °C temperature conditions were selected with the objective of studying differential protein expression between metabolite (picroside-I) accumulating and non-accumulating conditions. It has been validated experimentally that the amount of picroside-I obtained from shoots of 15 ± 1 °C maintained plant samples is ~17 times more than obtained from the shoots of 25 ± 1 °C maintained plant samples and shoot biomass is ~2 times more in case of 15 ± 1 °C *P. kurroa* as compared to 25 ± 1 °C *P. kurroa* [259]. Densitogram analysis

of the SDS-PAGE gel (Figure 4.4 (b)) confirms that both the number as well as the intensity of protein bands was greater in case of 15 ± 1 °C shoot samples as compared to 25 ± 1 °C shoot samples when the same amount of protein was loaded onto the gels. This finding is in accordance with the fact that the amount of picroside-I is greater in 15 ± 1 °C Shoots (2.03mg/g fresh shoot wt.) as compared to 25 ± 1 °C Shoots (0.12 mg/g fresh shoot wt.) as determined by HPLC [259]. The elevated levels of proteins as a consequence of lower temperature can be explained by the fact that when plants are under stress they tend to produce a variety of defense-related compounds known as phytoalexins which are not produced under normal conditions. Phytoalexins fall under various categories and one of these categories is terpenoid and we know that picroside is a terpenoid. So, if picroside is being formed under stress conditions the proteins responsible for it have to be overexpressed or uniquely expressed. Therefore, the objective of present study was to look for proteins that were overexpressed or uniquely expressed under stress condition (15 ± 1 °C). A study has shown that growing the plant at a temperature other than 15 ± 1 °C results in negligible amount of picroside-I synthesis [259]. Comparing the protein profiles between these two differential conditions will enable us to identify the enzymes that are overexpressed or uniquely expressed in 15 ± 1 °C shoot samples thus allowing us to identify the proteins involved in the picroside-I biosynthetic pathway.

MASCOT analysis of the MALDI-TOF MS results gave four interesting proteins out of eight that were over-expressed in case of 15 ± 1 °C shoot samples. These up-regulated proteins of interest were identified as NAD(P)H-quinone oxidoreductase subunit K (band 5), shikimate kinase (band 6), ribulose bisphosphate carboxylase small chain (band 7), fructokinase fragment (band 8).

Ribulose bisphosphate carboxylase is involved in carbon fixation (reductive pentose phosphate cycle/Calvin-Benson-Bassham Cycle). As can be seen in Figure 5.1, it catalyzes the first step of carbon fixation cycle (carboxylation of ribulose-1,5-bisphosphate to 3-phosphoglycerate). This 3-phosphoglycerate is then converted by phosphoglycerate kinase to glycerate-1,3-bisphosphate which is then converted to glyceraldehyde-3-phosphate by glyceraldehyde-3-phosphate dehydrogenase. This glyceraldehyde-3-phosphate so formed is fed into the MEP (non-mevalonate) pathway/chloroplast DOXP pathway [267] which might result in increased monoterpene synthesis and chlorophyll synthesis both of which are formed via the chloroplast DOXP pathway (Figure 5.1).

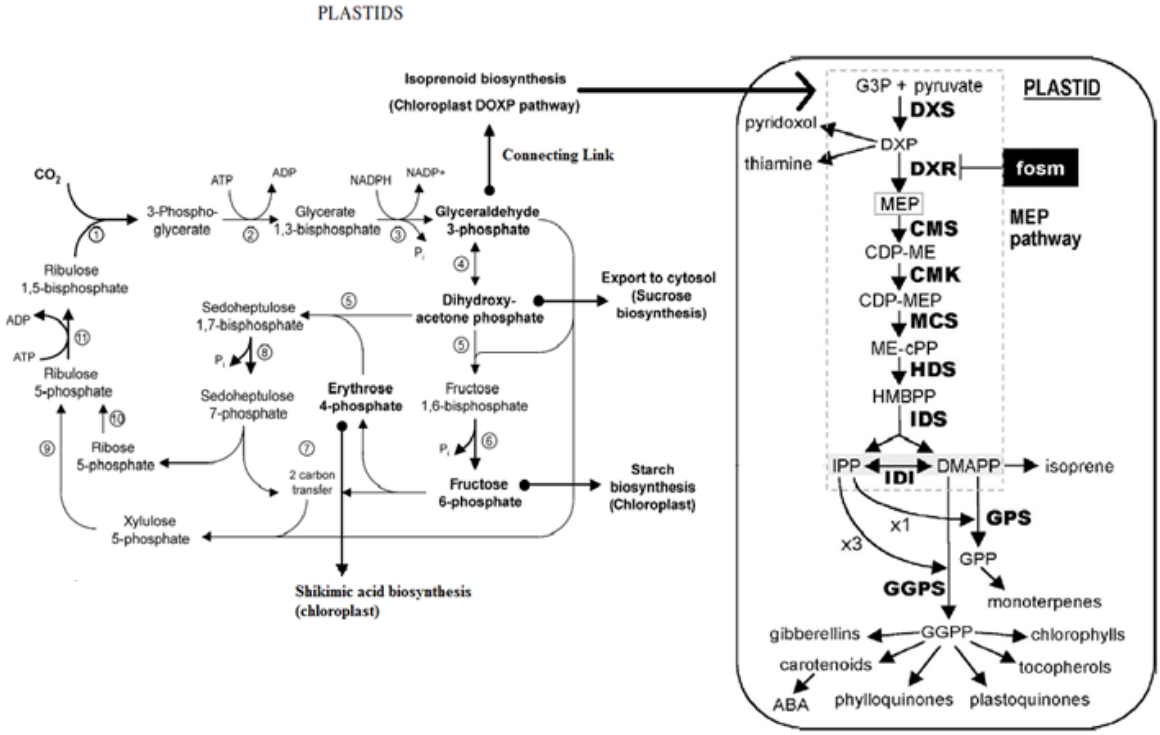


Figure 5.1 Calvin-Benson-Bassham Cycle and its link to MEP pathway: Rubisco (1), phosphoglycerate kinase (2), glyceraldehyde-3-phosphate dehydrogenase (3), triose-phosphate isomerase (4), aldolase (5), fructose 1,6-bisphosphatase (6), transketolase (7), sedoheptulose 1,7-bisphosphatase (8), ribulose phosphate epimerase (9), ribose 5-phosphate isomerase (10) and phosphoribulokinase (11) [9, 267]

It has also been shown that at 15 ± 1 °C the plants have higher picroside amount as well as shoot biomass yield [259]. This explains the physiological difference between 15 ± 1 °C and 25 ± 1 °C maintained *P. kurroa* plants where the 15 ± 1 °C plants are healthier and greener and also gives an explanation for the presence of higher picroside-I level in 15 ± 1 °C maintained plants as compared to 25 ± 1 °C plants.

Fructokinase, another up-regulated enzyme in case of 15 ± 1 °C shoot sample also helps to explain the observed physiological difference between both the plants, where 15 ± 1 °C plants are healthier than the 25 ± 1 °C plants. It is well documented that fructokinase is involved in starch synthesis. Sucrose is metabolized by sucrose synthase and/or invertase to form a hexose pool within which fructose is phosphorylated to fructose-6-phosphate by fructokinase and used in the biosynthesis of starch [268]. Fructose-6-phosphate is converted to glucose-6-phosphate by phosphohexose isomerase followed by conversion of glucose-6-phosphate to glucose-1-phosphate (the starting material for

starch synthesis) by phosphoglucomutase. It has been found that suppressing the synthesis of fructokinase isozyme Frk2 results in growth inhibition of stems and roots and reduction of flower and fruit number [268].

Shikimate kinase catalyzes the fifth step of the shikimate pathway where it phosphorylates shikimate to form shikimate-3-phosphate using ATP. The plant shikimate pathway leads the carbon from the central metabolism pool to a broad range of secondary metabolites involved in plant growth, development and stress responses [269]. Metabolites of the shikimate pathway are considered branch point substrates for other secondary metabolic pathways [270]. Shikimate pathway leads to the biosynthesis of phenylpropanoids [270]. Cinnamic acid and Vanillic acid from phenylpropanoid pathway have been recently shown to participate in the synthesis of picroside-I and picroside-II [254]. Thus, an overexpression of this enzyme reflects the synthesis of a higher amount of picrosides.

For optimal energy production plants possess additional pathways such as cyclic electron transport around photosystem I (PSI). NAD(P)H-quinone oxidoreductase subunit K is one of the 15 subunits of the NAD(P)H dehydrogenase (NDH) complex which is involved in photosystem I (PSI) cyclic and chlororespiratory electron transport in higher plants [271-273]. It shuttles electrons from NAD(P)H: plastoquinone, via FMN (flavin mononucleotide) and iron-sulphur (Fe-S) centres, to quinones in the photosynthetic chain and in a chloroplast respiratory chain. NDH helps in generating more ATP which can be used to sustain higher rates of biosynthesis of picroside-I.

b) 2-D gel electrophoresis

In this study, an endeavor was made to identify differentially expressed proteins in *P. kurroa* under the differential condition of picroside-I accumulation (15 ± 1 °C) and non-accumulation (25 ± 1 °C) and to correlate the results with the enhanced picroside-I content and observed phenotypic differences between the two samples. Proteomics analysis along with transcriptomics and metabolomics analysis has contributed a great deal to the understanding of the complex molecular mechanisms associated with a metabolic network of plants and how these networks respond to various factors. Medicinal plants for which fully sequenced and annotated genomes are not available, proteomic studies can help in exploring and investigating the various physiological pathways associated with metabolism, defence, signalling, and energy metabolism of the plants [274]. It has been experimentally validated that picroside-I content is ~17 times higher in 15 ± 1 °C

shoots (2.03 mg/g fresh shoot wt.) as compared to 25 ± 1 °C shoots (0.12 mg/g fresh shoot wt.) and the shoot biomass is approximately 2 times more in 15 ± 1 °C (3.73 ± 0.5 g) as compared to 25 ± 1 °C (2.03 ± 0.3 g) [259]. The enhanced picroside content can be accredited to the fact that plants grown at 15 ± 1 °C experience stress in contrast to plants grown at 25 ± 1 °C. Research has shown that stressed plant produces a variety of defence-related compounds known as phytoalexins [86]. Phytoalexins fall under various categories and one of these categories is terpenoids and we already know that picroside is a terpenoid [86]. So, if picroside is being synthesized under stress conditions, analyzing the proteome of stressed *P. kurroa* can help in unraveling the proteins involved in the biosynthesis of picrosides. Also, the plants that were grown at 15 ± 1 °C appeared to be greener and exhibited enhanced shoot biomass in comparison to plants grown at 25 ± 1 °C. Differential proteomics study can help us to account for these observed variations. When the same amount of protein was loaded, differential protein map was obtained for both the samples. Based on their biological functions, the differentially expressed proteins were classified into distinct functional categories by searching on UniProt database maintained at http://www.uniprot.org and KEGG database. Some of the proteins were identified in multiple spots. This phenomenon can be attributed to the fact of both proteins having the same experimental pI and molecular weight. There have been earlier reports of the presence of a protein in more than one spot suggesting the existence of post-translational modifications or isoforms [274].

Numerous proteins associated with secondary metabolic processes were found to be up-regulated or uniquely expressed in 15 ± 1°C shoot samples which is in congruence with previous reports [274, 275]. Proteomes of very few medicinal plants like *Chelidonium majus*, *Taxus cuspidate*, *Papaver somniferum*, *Catharanthus roseus*, and ginseng have been explored. For these plants, rich but limited sequence information is available [274]. Under such circumstances, proteome analysis can be performed by utilizing homology-based searches to proteins already in the database [274]. This method suffers from the limitation of identifying highly conserved proteins such as those participating in the primary metabolism but not essentially the proteins associated with specific secondary metabolic pathways like picroside-I biosynthesis which are specific to distinct plant species.

Although both MEP and MVA pathways are involved in the biosynthesis of picrosides, enzyme inhibitor studies with fosmidomycin (inhibits DXPR enzyme of MEP) and mevinolin (inhibits HMGR enzyme of MVA) revealed that MEP pathway is the

predominant pathway involved in picroside biosynthesis. Inhibition of MEP pathway resulted in 90.6% reduction in picroside-I accumulation compared to a decrease of only 17% when MVA pathway was inhibited [276].

It has been widely reported that primary and secondary metabolic pathways share an intimate relationship [277]. The scarce knowledge about the biosynthetic pathway involved in the biosynthesis of picroside-I encouraged us to explore this intimate relationship which exists in the form of feeder pathways for secondary metabolic pathways wherein primary metabolic pathways feed intermediate metabolites at various points of the secondary metabolic pathway (Figure 5.2).

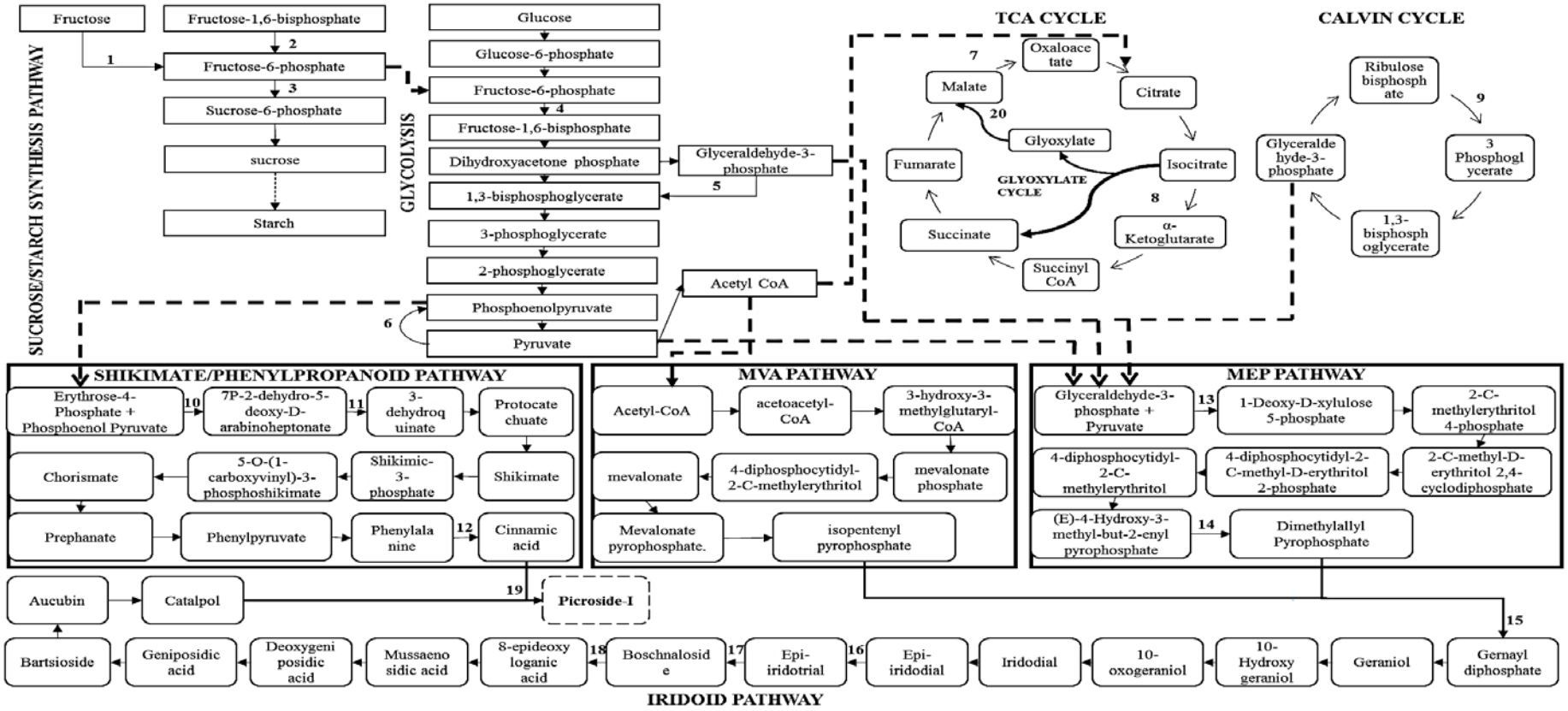


Figure 5.2 Intricate relationship between primary metabolic and picroside-I biosynthetic pathways. The reconstructed network includes pathways like sucrose and starch biosynthetic pathway, TCA cycle, Calvin cycle, glycolysis, MVA pathway, MEP pathway, iridoid pathway and phenylpropanoid/shikimate pathway. The numbered arrows depict the proteins identified to be associated with picroside-I biosynthesis. 1) FK, 2) FBpase, 3) SPS, 4) PFK, 5) G3PH, 6) PPDK, 7) MDH, 8) ISDH, 9) RubisCO, 10) DAHP synthase, 11) DQS, 12) PAL, 13) DXPS, 14) HMB-PP, 15) GGPS, 16) CYP, 17) UGT, 18), ADH 19) A5AAT and 20) MS

Metabolism related proteins

Metabolism related proteins constituted the largest fraction of the identified proteins. In the present study, up-regulation of two enzymes, 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) and 1-hydroxy-2-methyl-2-(E)-butenyl-4-phosphate reductase (ISPH) unique to the MEP pathway also showed the crucial role of MEP pathway in picroside biosynthesis. DXPS catalyzes the first and one of the rate-limiting steps of the MEP biosynthetic pathway wherein it synthesizes 1-deoxy-D-xylulose 5-phosphate from D-glyceraldehyde 3-phosphate and pyruvate. ISPH, the last enzyme involved in the MEP pathway converts 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate to isopentenyl diphosphate (IPP)/dimethylallyl diphosphate (DMAPP). Most terpenoids are made through the head-to-tail or head-to-head condensation of IPP and DMAPP [278].

In the picroside biosynthetic pathway, the IPP and DMAPP obtained above are further converted to catalpol via the iridoid pathway. Several enzymes of the iridoid pathway for example heterodimeric geranylgeranyl pyrophosphate synthase (GGPS) large subunit 1, geraniol 8-hydroxylase (G8H), cytochrome P450 monooxygenase (CYP), UDP-glycosyltransferase 73B3 (UGT), Aldehyde dehydrogenase (ADH) and Anthocyanin-5-aromatic acyltransferase (A5AAT) were observed to be up-regulated or uniquely expressed in 15 ± 1 ℃ shoots. The enhanced expression of these enzymes in relation to elevated picroside-I levels ascertains their participation in picroside biosynthesis and provides further evidence at the protein level to the picroside biosynthetic pathway reported by Shitiz et al., 2015 [276].

GGPS large subunit 1 is involved in step 1 of the subpathway that primarily catalyzes the conversion of IPP and DMAPP to geranylgeranyl pyrophosphate while the small subunit alone is inactive. Association of the two subunits alters the product profile to produce elevated levels of geranyl diphosphate [279], the precursor molecule for the formation of picrosides.

G8H, previously known as geraniol 10-hydroxylase [280], catalyzes the hydroxylation of geraniol to form 8-hydroxygeraniol which is highly similar to a step in the iridoid pathway of picroside biosynthesis catalyzed by geraniol 10-hydroxylase where instead of 8-hydroxygereniol it forms 10-hydroxygereniol.

CYPs are a diverse collection of enzymes which catalyze hydroxylation reactions. This enzyme has been reported to be involved in the iridoid pathway of picroside biosynthesis where it catalyzes the conversion of epi-iridodial to epi-iridotrial [276].

UGT is pivotal in the process of glycosylation for decorating natural products with sugars [51]. A UGT has been reported to participate in the conversion of epi-iridotrial to boschnaloside in the iridoid pathway of picroside biosynthesis [276].

ADH possesses activity on acetaldehyde and glycolaldehyde in vitro. In picroside biosynthetic pathway, this enzyme has been proposed to be involved in the iridoid pathway where it catalyzes the conversion of boschnaloside to 8-epideoxy loganic acid [276].

A5AAT transfers hydroxycinnamic moieties to the glucosyl groups of anthocyanin. It has been proposed to participate in the final step of picroside biosynthesis wherein catalpol is esterified with either cinnamic acid to form picroside-I or vanillic acid to form picroside-II.

Shikimate and phenylpropanoid biosynthetic pathways are crucial to the biosynthesis of picrosides. The cinnamic acid and vanillic acid required during the final steps of picroside-I and picroside-II biosynthesis respectively are synthesized using these pathways. Several enzymes of these pathways like 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHPS), 3-dehydroquinate synthase (DQS), Phenylalanine ammonia-lyase (PAL), caffeic acid 3-O-methyltransferase (CAM) were found to be showing enhanced expression in 15 ± 1 °C shoots. DAHPS detected in two distinct spots is a rate-limiting enzyme of the shikimate pathway [281]. DAHPS, the first enzyme of the shikimate pathway, controls the amount of carbon entering the pathway. This pathway diverts carbon from the central metabolic pool for producing a variety of secondary metabolites which are involved in plant development, growth and stress responses [269]. Elevated expression of DQS, another enzyme of the shikimate pathway which catalyzes the second step of converting 3-deoxy-d-arabino-heptulosonate-7-phosphate into 3-dehydroquinate further substantiated the role of shikimate pathway in picroside biosynthesis. The shikimate pathway is a gateway for entry into the phenylpropanoid pathway [282], which is the source of cinnamic acid required for picroside-I biosynthesis along with catalpol derived from the iridoid pathway [283].

PAL, the first committed enzyme of phenylpropanoid pathway which converts phenylalanine to cinnamic acid was also found to exhibit enhanced expression indicating its participation in the formation of picroside-I. Another enzyme of the phenylpropanoid pathway, CAM, involved in the production of vanillic acid, required for picroside-II biosynthesis was also observed to be up-regulated in 15 ± 1 °C shoots. This vanillic acid can be transported from here by an ABC transporter to either the roots or stolons where it can begin participating in picroside-II biosynthesis.

It has been widely reported that primary and secondary metabolic pathways share an intimate relationship [277]. The scarce knowledge associated with picroside-I biosynthetic pathway encouraged us to explore this intimate relationship which exists in the form of feeder pathways for secondary metabolic pathways wherein primary metabolic pathways feed intermediate metabolites at various points of the secondary metabolic pathway (Figure 5.2).

The present study identified the overexpression of proteins (Table 4.3) participating in crucial primary metabolic pathways like TCA cycle and glycolysis. These pathways, feed metabolites both into primary metabolic processes such as pentose phosphate pathway and secondary metabolic pathways such as MEP (2C-methyl-d-erythritol 4-phosphate), MVA and shikimate/phenylpropanoid pathway to produce picrosides (Figure 5.2).

Numerous enzymes associated with primary metabolic processes were observed to be uniquely expressed or up-regulated in 15 ± 1°C shoots. These enzymes were identified as isocitrate dehydrogenase [NADP] (ISDH), malate synthase (MS), malate dehydrogenase [NADP] (MDH), glyceraldehyde-3-phosphate dehydrogenase (G3PH), ribulose bisphosphate carboxylase (RuBPCase) small chain, fructose-1,6-bisphosphatase (FBPase), ATP-dependent 6 phosphofructokinase (PFK), fructokinase-6 (FK), pyruvate, phosphate dikinase (PPDK) and sucrose phosphate synthase (SPS). The enzymes mentioned above were found to be involved in some primary metabolic processes like glycolysis, TCA cycle, Calvin cycle, gluconeogenesis, and starch biosynthesis. Secondary metabolite production is entwined with numerous primary metabolic pathways such as shikimate pathway, TCA cycle, glycolysis, pentose phosphate pathway and those involved in the production of aliphatic and aromatic amino acids [284].

A crucial role is played by G3PH under stress conditions in addition to catalyzing the oxidation of triose phosphates during glycolysis [285-287]. It transduces the ROS hydrogen peroxide signal by interacting with phospholipase D associated with the plasma membrane [285]. It is presumed that it enhances the malate-valve capacity and the effect of other protective systems by being part of a signaling pathway [286]. This enzyme takes part in energy production and siphons of various intermediates for cellular metabolism [288]. Overexpression of G3PH indicates an upsurge in the reversible reaction of converting glyceraldehyde-3-phosphate (G3P) to 3-phosphoglycerate (3PG) and vice versa representing an increased influx of G3P for siphoning into the MEP pathway or flux of 3PG

further into the glycolysis for the formation of pyruvate. The latter also being the starting material for MEP pathway [289].

Ribulose bisphosphate carboxylase (RuBPCase) participates in carbon fixation (Calvin cycle). RuBPCase is a rate-limiting enzyme and participates in the first major step of carbon fixation cycle where it catalyzes the formation of 3PG by carboxylating ribulose-1,5-bisphosphate. 3PG is further converted by phosphoglycerate kinase to glycerate-1,3-bisphosphate which is then converted by G3PH to G3P. This G3P is syphoned into the MEP pathway [290] which can account for the observed increase in monoterpene synthesis (picroside-I) and chlorophyll synthesis (15 ± 1 °C plants greener than 25 ± 1 °C plants) [290].

FBPase is involved in Calvin cycle and starch biosynthesis. Reduction in FBPase activity has been shown to cause a decrease in growth and photosynthetic activity [291]. Its up-regulation in 15 ± 1 °C shoots can help explain the reason of 15 ± 1 °C shoots being healthier than 25 ± 1 °C shoots (Figure. 5.3).

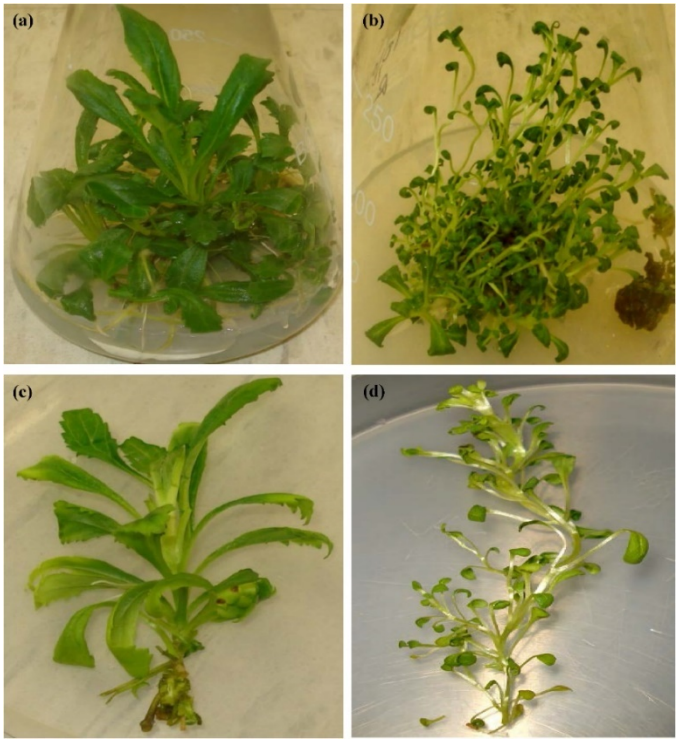


Figure 5.3 Phenotypic differences among shoots of tissue cultured *P. kurroa* plants. Plants grown at 15 ± 1 °C (a and c) were found to possess healthier shoots and were well developed as compared to 25 ± 1 °C (b and d) [53]

Increased expression of FK reflects an increase in production of fructose-6-phosphate (F6P). This F6P can be acted upon by SPS, another enzyme that was found to be up-regulated in 15 ± 1 °C shoots, for the formation of sucrose-6-phosphate which is ultimately converted to sucrose to be stored as starch allowing the plant to survive under cold temperature stress conditions.

PFK is an enzyme which catalyzes the addition of a phosphate group from ATP to D-fructose 6-phosphate to form fructose-1,6-bisphosphate, the first committing step of glycolysis. Its enhanced expression means higher availability of pyruvate and G3P to be fed into the MEP pathway.

A TCA cycle enzyme, ISDH, which is a rate-limiting enzyme was also found to be overexpressed. Its overexpression does not necessarily reflect its involvement in picroside-I biosynthesis but could be involved in producing a higher concentration of TCA cycle intermediates and ATP required for the growth and development of *P. kurroa*. ISDH catalyzed reaction being one of the irreversible steps is supposed to be a key regulatory point of the TCA cycle [292]. Experiments with mutants lacking gene encoding for isocitrate dehydrogenase exhibited a reduced growth phenotype [292].

Malate synthase (MS) participates in the glyoxylate cycle where it catalyzes the formation of malate and CoA. It allows replenishing the pool of TCA cycle intermediates necessary for gluconeogenesis and other biosynthetic processes [293]. Overexpression of MS can be corroborated to enhanced levels of malate dehydrogenase (MDH) which converts malate to oxaloacetate or vice versa in the TCA cycle. An increase in the activity of MDH is expected to influence the biosynthesis of picrosides by generating intermediates of TCA cycle in increased concentration in addition to NADH which could be employed for ATP production [294]. Compared to *Arabidopsi*s mutants lacking malate synthase the wild-type Arabidopsis used their lipid more rapidly and were better able to establish as plantlets [295].

Pyruvate, phosphate dikinase, a key enzyme functioning in gluconeogenesis and photosynthesis, catalyzes the conversion of pyruvate to phosphoenolpyruvate (PEP). PEP is a starting material of shikimate pathway required for the production of cinnamic acid via phenylpropanoid pathway for picroside-I biosynthesis.

SPS plays a major role in photosynthetic sucrose synthesis by catalyzing the rate-limiting step of sucrose biosynthesis from UDP-glucose and F6P. It may regulate the synthesis of sucrose and therefore play a major role as a limiting factor in the export of photo-assimilates out of the leaf. It is also involved in maintaining sucrose availability which is an essential

requirement for plant growth. Mutants with the loss of SPS function have been shown to exhibit a reduction in plant growth [296].

In addition to the above-mentioned proteins, numerous other proteins performing critical functions in plants were observed to exhibit elevated or unique expression in 15 ± 1°C shoots. Below is a reference of some of these imperative proteins.

3-hydroxyisobutyryl-CoA hydrolase has been reported to play an important role in cold signalling and cold tolerance [297]. Radical superfamily protein provides evidence that radical-based catalysis is important in many previously well- studied but unresolved biochemical pathways and reflects an ancient conserved mechanistic approach to difficult chemistries [298]. Jasmonate O-methyltransferase catalyzes the methylation of jasmonate into methyl jasmonate, a plant volatile that acts as an important cellular regulator mediating diverse developmental processes and defence responses [299]. Caffeoyl-CoA O-methyltransferase, involved in the reinforcement of the plant cell wall confers additional protection to the plants against various biotic as well as abiotic stresses [300]. Cytochrome b-c1 complex subunit Rieske-4, component of the electron transport chain that generates an electrochemical potential coupled to ATP synthesis, ensures the availability of surplus energy for the plant during adverse conditions. 9-cis-epoxycarotenoid dioxygenase catalyzes a key step in abscisic acid (ABA) biosynthesis. ABA plays a major role in adaptation to environmental stresses and regulation of growth and development [301]. cinnamyl alcohol dehydrogenase is involved in lignin biosynthesis. These polymers are deposited primarily in the walls of secondarily thickened cells, making them rigid and impervious. Lignin biosynthesis is reported to be induced in response to various biotic and abiotic stress conditions to confer protection to plants [302].

Proteins related to transcription and translation

In 15 ± 1°C shoots ribosomal protein-small subunit 4 was found to be uniquely expressed while ribosomal protein S11 was found to be down-regulated. Ribosomal proteins (RPs) are well-known for their role in mediating protein synthesis and maintaining the stability of the ribosomal complex [303]. Phytohormone and cold treatments induced significant up-regulation of several RPL genes, while heat and H_2O_2 treatments down-regulated a majority of them. The expression of RPL genes is highly responsive to stress and signalling molecules indicating that their encoded proteins appear to have roles in stress amelioration besides house-keeping. This shows that the RPL gene family is a valuable resource for

manipulation of stress tolerance in rice and other crops [303]. Another uniquely expressed protein, NAC domain-containing protein 21/22 is a transcriptional activator that mediates auxin signalling to promote lateral root development. It is known to activate the expression of two downstream auxin-responsive genes, DBP and AIR3 [304]. This might explain the extensive root network observed in 15 ± 1°C *P. kurroa* as compared to 25 ± 1°C *P. kurroa*. DNA polymerase epsilon subunit 3, a protein observed to be up-regulated, participates in chromosomal DNA replication. It is essential for the proper shoot (SAM) and root apical meristem (RAM) functions. It is also involved in maintaining epigenetic states, controlling hypersensitive response (HR), and mediating abscisic acid (ABA) signalling [305-308]. Its main role may be in the progression of the CMG complex to unwind the replication fork and in the repair of replication errors [309]. Plants under stress have been recognized to suffer from defects in chloroplast translation [310, 311]. The down-regulation of zinc-finger homeodomain protein 3 was observed to occur in 15 ± 1°C *P. kurroa* shoots. Homeodomain proteins are known to play important roles in plant development [312]. Mutant analyses from monocot and dicot plants showed that especially the formation of leaves involves downregulation of meristem-specific homeobox genes [313]. This explains why 15 ± 1°C *P. kurroa* has enhanced shoot biomass.

Proteins related to signal transduction

Histidine-containing phosphotransfer protein 1-like isoform X1 which was uniquely expressed in 15 ± 1°C *P. kurroa* shoots functions as a two-component phosphorelay mediator between cytokinin sensor histidine kinases and response regulators (B-type ARRs). It plays an important role in propagating cytokinin signal transduction through the multistep His-to-Asp phosphorelay. They mediate the signal from sensory kinases (usually membrane proteins) to response regulators in the nucleus in response to environmental stress or a hormone signal [314]. Two-component response regulator ARR15-like isoform X2 another protein found to be uniquely expressed functions as response regulator involved in His-to-Asp phosphorelay signal transduction system. Phosphorylation of the Asp residue in the receiver domain activates the ability of the protein to promote the transcription of target genes [315, 316]. The significance of ankyrin repeat domain-containing protein 65, the third uniquely expressed protein of this category reflects from the fact that it is an integral part of numerous proteins of diverse function such as transcriptional initiators, cell-cycle regulators, cytoskeletal, ion transporters and signal

transducers [317]. Another important protein glycogen synthase kinase (GSK)-3 homolog MsK-3 was observed to be up-regulated. These are multifunctional non-receptor ser/thr kinases. Plant GSKs are involved in hormonal signalling networks and are required for growth, development, light as well as stress responses [318]. Three serine/threonine-protein kinases exhibiting differential expression were identified. One was found to be uniquely expressed, the second one up-regulated and the third one was down-regulated in 15 ± 1°C *P. kurroa* shoots. The network of protein serine/threonine kinases in plant cells appears to act as a "central processor unit" (CPU), accepting input information from receptors that sense environmental conditions, phytohormones, and other external factors, and converting it into appropriate outputs such as changes in metabolism, gene expression, and cell growth and division. These enzymes are involved in a variety of responses such as (a) response to soluble extracellular messenger molecules, (b) response to messenger molecules anchored in the surface of neighbouring cells, (c) defense responses, (d) the timing of events that occur discontinuously in the cell cycle and (d) response to stressful environment conditions [319].

Proteins related to stress and defence

Proteins belonging to this category exhibited enhanced expression in 15 ± 1°C shoots indicating their importance in the survival of the stressed *P. kurroa* grown at 15 ± 1°C. The imperative proteins identified as a molecular chaperone (DnaJ superfamily) (ISS), chaperonin 60 subunit beta, late embryogenesis abundant protein 47 and protein COLD-REGULATED 15B were observed to be uniquely expressed. Stress conditions that influence protein folding dynamics within a cell can lead to changes in expression of the components of the protein folding quality control system [320]. Molecular chaperones function in a variety of protein biosynthetic events and protect proteins from the deleterious effects of acute or chronic stress by stabilizing and refolding protein-folding intermediates or facilitating protein degradation [320]. Molecular chaperone (DnaJ superfamily) (ISS) in addition to its essential role in the degradation of abnormal proteins, assists in the folding of nascent polypeptides, their translocation across membranes, and the assembly of oligomeric complexes [321, 322]. Chaperonin 60 subunit beta in addition to its role in protecting plant under stress is also involved in the assembly of RuBisCO small and large subunits for protecting photosynthesis during stress conditions [323] and required for the formation of a normal plastid division apparatus [324].

Chaperonin 60 subunit beta has been shown to be up-regulated during stress [325]. Late Embryogenesis Abundant (LEA) proteins, a group of hydrophilic proteins, have been linked to survival in plants and animals in periods of stress, putatively through safeguarding enzymatic function and prevention of aggregation in times of dehydration/heat. Many proteins, including the enzymes citrate synthase and lactate dehydrogenase, form insoluble aggregates when dried or frozen, but aggregation is reduced in the presence of LEA proteins. LEA proteins might act as hydration buffers, slowing down the rate of water loss during dehydration; during partial drought, osmotic or chilly stress, hydration buffers allow sufficient water activity for proteins to retain function. The anti-aggregation activity of LEA proteins resembles a "holding" molecular chaperone, which function in the cell would be to stabilize passively protein species in a partially unfolded state, preventing aggregation while the stress lasts. They resemble holding chaperones, in their functioning without ATP, in contrast to classical folding chaperones which require ATP [326]. protein COLD-REGULATED 15B Exhibits cryoprotective activity toward stromal substrates in chloroplasts and in protoplasts and confers freezing tolerance to plants in a CBF-dependent manner. It functions as a protectant against various stresses (e.g. cold, drought and heat stress) by preventing protein aggregation and attenuating enzyme inactivation. It Influences the intrinsic curvature of the inner membrane of the chloroplast envelope and modulates the freeze-induced lamellar-to-hexagonal II phase transitions that occur in regions where the plasma membrane is brought into close apposition with the chloroplast envelope during freeze-induced osmotic contraction [327]. It mediates a shift in the melting curves of phospholipids-containing membranes to lower temperatures. It is also involved in the regulation of leaf senescence by abscisic acid (ABA) in a VNI2-dependent manner [328].

Proteins related to transport

Four ABC transporter family proteins were found to show enhanced expression in 15 ± 1°C shoots. The membrane transport of plant secondary metabolites is a newly developing research area. It has been found that ATP-binding cassette (ABC) transporters are involved in the transport of these secondary metabolites in some plant systems. Not only genes that are involved in the biosynthesis of secondary metabolites but also those that are involved in their transport will be important for systematic metabolic engineering aimed at increasing the productivity of valuable secondary metabolites in plants [329]. Storage vacuoles, which often occupy 40–90% of the inner volume of plant cells, play a

pivotal role in the accumulation of secondary metabolites in plants. The accumulation of secondary metabolites in vacuoles has at least two positive roles: the sequestration of biologically active endogenous metabolites inside the cells and the protection of such metabolites from catabolism [330]. Two major mechanisms are proposed for the vacuolar transport of secondary metabolites: H+-gradient-dependent secondary transport via H+-antiport and directly energized primary transport by ATP-binding cassette (ABC) transporters [331]. Expression of these transporters under conditions of picroside accumulation indicates their potential role in the transport of picrosides from their site of biosynthesis to their site of storage in vacuoles.

Proteins related to cell cycle

Stress conditions lead to multiple disorganizations of the cell ultrastructure. Stress results in a decrease in a number of dividing cells and a change in the relationship between cell cycle phases. Also, increased cell differentiation of some cells has been observed. Cyclin-dependent protein kinase inhibitor SMR3-like isoform X1 was found to be down-regulated in 15 ± 1°C shoots. It functions as a repressor of mitosis in the endoreduplication cell cycle. Cyclin-Dependent Kinase Inhibitors SMR5 regulate the DNA damage checkpoint in response to reactive oxygen species [332].

Proteins with unknown function

A number of proteins with unknown functions were identified in our study. These proteins comprised of peptides obtained from theoretical translation of nucleotide sequences which have never been identified as protein products. More intensive work is needed to characterize these proteins so as to establish their role in *P. kurroa*.

5.3 Differential protein expression studies in *P. kurroa* to investigate the presence of proteins involved in the biosynthesis of picroside-I and picroside-II in stolons

It has been experimentally validated that both picroside-I and picroside–II are present in Sairopa stolons as compared to 15 ± 1 °C root samples. The estimation of P-I and P–II content in different tissues helped in the identification of differential conditions for the biosynthesis of P-I and P-II. The higher content of P-I and P-II in case of Sairopa stolons can be attributed to various climatic issues such as light, temperature, altitude, UV, etc. [71]. When the same amount of protein (15µg) was loaded onto the gel,

differentially expressed proteins were identified in Sairopa stolon. These differentially expressed proteins were categorized according to their biological functions by searching on UniProt database at http://www.uniprot.org.

Stress-related proteins

Sairopa samples from high altitudes and grown in the greenhouse were under more stress as compared to 15 ± 1 °C samples grown using tissue culture. This resulted in a number of stress-related proteins being uniquely expressed in Sairopa sample. Methionine sulfoxide reductase the reduction of methionine sulfoxide to methionine which is oxidized to methionine sulfoxide under oxidation conditions. This results in a change in protein hydrophobicity and it's folding ultimately affecting its catalytic purpose [333, 334]. For example, a heat shock protein, Hsp21 loses its chaperone activity when methionine residues are oxidized. The action of methionine sulfoxide reductase helps in attaining a fully active enzyme by reduction of these oxidized methionine residues using thioredoxin as the reductant [333]. Peptidyl-prolyl cis-trans isomerase also known as conformases or rotamases are involved in protein folding because of their ability to catalyze slow steps in the initial folding/rearrangement of proteins [335, 336]. Peptidyl-prolyl cis-trans isomerase functions to prevent or reverse protein aggregation resulting from stress conditions [336]. DnaJ is part of the DnaK−DnaJ chaperone system which is centrally involved in heat stress response in response to destabilizations which cause protein misfolding [337]. Although DnaJ can act as a chaperone on its own, it generally functions as a co-chaperone with DnaK [338]. Glyceraldehyde-3-phosphate dehydrogenase catalyzes the oxidation of triose phosphates during glycolysis. In addition to this role, it is also involved in stress conditions [285, 339] where it has been shown to interact with the plasma membrane-associated phospholipase D to transduce the ROS hydrogen peroxide signal [285]. It is assumed that it acts as a part of the signalling pathway to increase malate-valve capacity and the effect of other protective systems [339]. 1-aminocyclopropane-1-carboxylate oxidase is involved in ethylene biosynthesis in response to various biotic and abiotic stress conditions [340]. It catalyses the oxidation of 1-aminocyclopropane-1-carboxylate (ACC) to ethylene [341]. Previous studies showed that changes in monoterpene concentration are generally related to the rate of ethylene production, that is, with high rates of ethylene production monoterpene concentrations were also

found to be increased [342]. Therefore, an overexpression of this enzyme indicated a possibility of its involvement in picroside biosynthesis. Chaperone DnaJ like protein is involved in protein folding and assembly. Different DnaJ-like proteins interact with specific Hsp70s forming pairs adapted to each other and function as chaperone system protecting plants against various stress conditions [343].

Signaling pathways related proteins

Plants require vesicular transportation for various specialized phenomena and common housekeeping events. Rab GTPase is the key player involved in vesicular transport. They act as molecular switches controlling the fusion of vesicles with target membranes via transition between GTP and GTP-bound forms [344].

Metabolic pathway related proteins

Glyceraldehyde-3-phosphate dehydrogenase is associated with glycolysis where it catalyzes the reversible reaction of converting glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate [285, 344]. This enzyme is involved in energy production and syphoning of various intermediates for cellular metabolism [344]. Overexpression of this enzyme leads to the production of more pyruvate which is one of the starting molecules of MEP pathway involved in the biosynthesis of picrosides (Figure 5.4) [345].

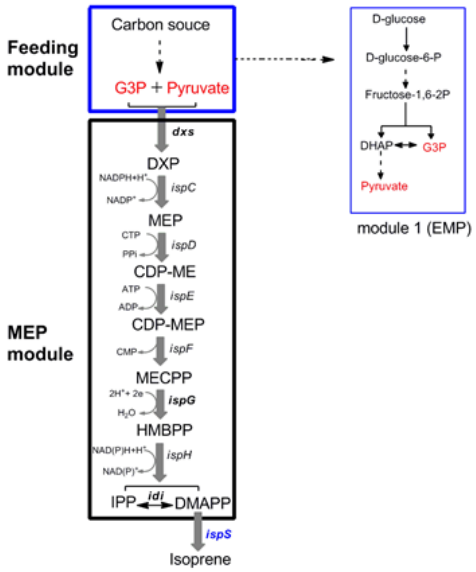


Figure 5.4 Connecting link of MEP pathway to glycolysis for the supply of glyceraldehyde-3- phosphate, and pyruvate, the two starting molecules of MEP pathway. Module 1 i.e. glycolysis is of interest as the overexpressed enzyme Glyceraldehyde-3-phosphate

dehydrogenase is involved in this pathway. Gene symbols and the enzymes they encode: *dxs*, DXP synthase; *ispC*, DXP reductoisomerase; *ispD*, DXP-ME synthase; *ispE*, CDP-ME kinase; *ispF*, MECPP synthase; *ispG*, HMBPP synthase; *ispH*, HMBPP reductase; *idi*, IPP isomerase; *ispS*, isoprene

Phospholipids are responsible for maintaining the epidermal permeability barrier. This barrier prevents transcutaneous water loss helping in plant survival. 1-acyl-sn-glycerol-3-phosphate acyltransferase 2 is the crucial enzyme involved in the biosynthesis of phospholipids and triglycerides. This enzyme causes acylation of lysophospholipids to phosphatidic acid which is the major precursor of all phospholipids/triglycerides [346]. 2-oxoglutarate ferrous-dependent oxygenases is a superfamily of enzymes that are known to catalyze various reactions like hydroxylations, desaturations and oxidative ring closures [347]. They are involved in post-translational modification of collagen and in the biosynthesis of both primary and secondary metabolites [347] including flavonoid biosynthesis a kind of secondary metabolites derived from phenylalanine & acetate metabolism. 2-oxoglutarate ferrous-dependent oxygenases catalyze multiple steps within the same pathway owing to flexibility in metal coordination chemistry suggest its involvement in unusual reactions [347]. UbiE/COQ5 methyltransferase belongs to the family of methyltransferases which participate in the biosynthesis of menaquinone and ubiquinone. Ubiquinone is involved in the respiratory chain where it transfers an electron from complex I (or complex II) to complex III. Ubiquinone has been explored for roles other than in electron transfer such as its role in/as antioxidant, disulphide bond formation, extension of lifespan due to lack of ubiquinone [348]. All of these functions suggest its importance in survival. Cytochrome P450 enzymes are involved in various biosynthetic and detoxification pathways. Within biosynthetic pathways, these enzymes have played a tremendous role in the biosynthesis of lignin intermediates, sterols, terpenes, flavonoids, isoflavonoids, furanocoumarins and other secondary metabolites [349]. Cytochrome P450 dependent monooxygenases have been found to increase the structural diversity of terpenoids [350]. Adenylate isopentenyl transferase is involved in cytokinin biosynthesis where it catalyses the transfer of an isopentenyl group from dimethylallyl diphosphate (DMAPP) to N6 amino group of adenosine phosphate to produce isopentenyl adenosine phosphates [351, 352] which are then converted to isopentenyladenine and trans-zeatin [351]. These cytokinins are

involved in plant growth and development [352]. 3-ketoacyl-CoA synthase 11 is involved in the biosynthesis of cuticular wax and suberin [353]. Cuticle present on plant surfaces acts as the first line of defence against pathogens, insects and environmental stresses such as drought, UV damage and frost. Overexpression of this protein confers protection to *P. kurroa*. Differential expression of all the proteins in this category help in plant survival and in the formation of secondary metabolites.

Transcriptional and translational factors related proteins

Regulating genes at transcription level serves as one of the most important points of regulation in biological processes. Ethylene-responsive transcription factor WIN1 has been shown to be related to plant development, defence response and stress signalling pathways enabling plants in adjusting to their adverse surroundings [354]. It promotes cuticle formation by inducing the expression of enzymes involved in wax biosynthesis. It provides protection against drought resistance [355]. 40S ribosomal protein S13-1 is involved in the translation of mRNA to proteins which may help plants to synthesize new proteins or replace damaged proteins to help plants cope with numerous stress conditions. Overexpression of this protein indicated that stressed plant is undergoing heavy translation.

Energy metabolism-related proteins

Ferredoxin belongs to the family of oxidoreductases which use iron-sulphur proteins as electron donors and NAD^+ or $NADP^+$ as electron acceptors. These function primarily in photosynthesis where they transfer electrons from photo reduced Photosystem I to ferredoxin NADP(+) reductase in which NADPH is produced for CO_2 assimilation [356]. Ferredoxin can also function in removing excessive reducing power and preventing uncontrolled over-reduced states that are common in stroma under stress conditions [357]. Photosystem I reaction center subunit V of the photosystem I is an integral membrane protein [358]. It is involved in stabilizing the binding of peripheral antenna and regulation of Photosystem I [359]. Photosystem I function to produce NADPH necessary for the reduction of CO_2 in the Calvin-Benson-Bassham cycle. This cycle has been previously shown to be linked to MEP pathway for the synthesis of picrosides [345]. Photosystem I is involved in the cyclic synthesis of ATP from the light generating large amounts of ATP for sustaining various metabolic and physiological processes [360]. NADH dehydrogenase subunit F

is a part of NADH dehydrogenase. NADH dehydrogenase allows electron transport to continue even when the membrane potential is high, thus uncoupling electron transport from ATP synthesis. Removal of intermediates from the citric acid cycle as would happen during secondary metabolite synthesis requires NAD^+ to be recycled at a higher rate than coupled transport allows. This problem is solved by NADH dehydrogenase catalyzed recycling of NAD^+ in mitochondrial matrix [361].

Mitochondria play an important role in respiration and energy production and is involved in several plant metabolic pathways. Mitochondrial carrier family proteins connect the internal metabolism with that of the cells surrounding allowing the exchange of ATP, di- and tricarbonic acids basic amino acids, carnitine, S-Adenosylmethionine, phosphate and reducing power etc. from and to the mitochondria [362].

SUMMARY

P. kurroa is associated with innumerable medicinal properties most potent being the ability to treat various liver disorders. Herbal formulations meant for treating hepatic disorders use a particular ratio of picroside–I and picroside–II as the main active components. Therefore, it becomes all the more important to study their biosynthesis to understand their biosynthesis within *P. kurroa*. Studying difference in proteomes of samples corresponding to metabolite accumulation and non-accumulation can help in elucidating important proteins involved directly or indirectly in the biosynthesis of these potent molecules. Differential proteomic analysis can aid in profiling altered proteins enabling a better understanding of various physiological processes occurring in plants. In the present study, SDS-PAGE and 2-D gel electrophoresis combined with mass spectrometry-based protein identification revealed altered proteins belonging to several functional categories like metabolic pathways, stress response, signalling pathways, photosynthesis, cell cycle, transport, transcription and translation factors and energy metabolism. When the study was initiated no proteomics-based study had ever been done on this plant. Therefore, the first objective at hand was to identify the most efficient protein extraction method capable of extracting highest amount of protein from different tissues (shoots, roots, and stolons) of *P. kurroa*. Of all the extraction methods employed, Tris-Cl method succeeded in isolating the highest concentration of proteins from shoots and roots of *P. kurroa* while TCA-Acetone was successful in extracting maximum concentration of proteins from the stolons of *P. kurroa*. Previous studies have shown that shoots of tissue cultured *P. kurroa* maintained at 15 ± 1 °C have enhanced picroside-I content and biomass as compared to *P. kurroa* maintained at 25 ± 1 °C [259]. To identify the proteins responsible for these differences differential proteomics analysis was performed between shoots of 15 ± 1 °C and 25 ± 1 °C maintained *P. kurroa*. A total of 108 differentially expressed proteins were identified using SDS-PAGE and 2-D gel electrophoresis coupled with mass spectrometry-based identification of these differentially expressed proteins. In addition to identifying proteins associated with picroside-I biosynthesis and shoot biomass increase an intimate relationship between primary metabolic pathways and the picroside-I biosynthetic pathway was also revealed. The identified proteins expanded our insight into better understanding the various physiological processes of this plant. To investigate whether stolons are also involved in picroside

biosynthesis as both picroside-I and picroside-II are found in stolons, the differential proteomic analysis was done between stolons of *P. kurroa* plants obtained from Sairopa and 15 ± 1 °C roots which contain neither picroside-I nor picroside-II. Identification of certain metabolism-related proteins pointed to their potential role in picroside biosynthesis but a more thorough investigation utilizing an integrated omics approach is required to validate this claim.

At this time, the sequence information on *P. kurroa* is partially available. As this information becomes available, the proteome data will certainly prove to be a useful tool for the thorough understanding of the roles the proteins play under any set of given conditions. This work can act as a stepping stone for carrying further the research in the field of proteomics because no proteome data is available till date for this plant. It would be interesting to find out all of the remaining proteins thus enabling the generation of a complete proteome map of *P. kurroa*. In future, once all the enzymes involved in the biosynthesis of picrosides are identified and validated using proteomics study, the rate-limiting enzyme can be identified and engineered to increase the overall production of this metabolite along with an increase in shoot biomass − the two important traits of economic importance. Furthermore, MEP and MVA pathways involved in picroside biosynthesis are expressed in several other organisms like plants and microorganisms. Metabolic engineering of iridoid and phenylpropanoid pathway into these organisms will enable the acquisition of picrosides from entirely new organisms thereby reducing the overexploitation of *P. kurroa* for picrosides and thus preventing it from getting extinct.

CONCLUSION AND FUTURE PROSPECTS

The outcomes from this study helped in identifying imperative proteins responsible for the enhanced picrosides content and shoot biomass in *P. kurroa*. The differentially expressed proteins identified could help in getting a better insight into how the plant copes with stress condition and enhances picroside content and shoot biomass. The proteomic analysis of medicinal plants in the absence of fully sequenced and annotated genomes can allow exploration and investigation of physiological pathways related to metabolism, defence, signalling, and energy metabolism of these medicinal plants. The study also revealed an intimate relationship between various primary metabolic pathways and picroside biosynthetic pathway wherein primary metabolic pathways feed picroside biosynthetic pathway with various substrates increasing the overall yield of picrosides. Identification of certain secondary metabolism enzymes in stolons of *P. kurroa* obtained from Sairopa indicated their potential role in picroside biosynthesis but further studies utilizing an integrated omics approach is required to validate this claim. This is the first report on proteomics study in *P. kurroa* wherein proteins responsible for enhanced picroside level and shoot biomass have been identified. The expression of these proteins can be increased resulting in the enhanced picrosides formation and shoot biomass - the two desirable traits of economic importance. Once all the proteins of the pathway have been experimentally validated the pathway could be engineered into other organisms for the production of picrosides.

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LIST OF PUBLICATIONS

Publication in peer reviewed journals

1. **Sud A**, Chauhan RS and Tandon C. (2013). Identification of imperative enzymes by differential protein expression in *Picrorhiza kurroa* under metabolite accumulating and non-accumulating conditions. *Protein & Peptide Letters, 20*(7), 826-835. doi: 10.2174/0929866511320070014 **(Impact Factor 0.964)**
2. **Sud A**, Chauhan RS and Tandon C. (2014). Mass spectrometric analysis of differentially expressed proteins in an endangered medicinal herb, *Picrorhiza kurroa*. *Biomed Research International, 2014*, 326405. doi: 10.1155/2014/326405 **(Impact Factor 2.476)**
3. **Sud A**, Chauhan RS, Sood H and Tandon C. (2016). A proteomic approach to identify differentially expressed proteins in tissue cultured *Picrorhiza kurroa* shoots: An Endangered Medicinal Herb. (Communicated)

Publication in conference

1. **Sud A,** Chauhan RS and Tandon C. Correlation of differentially expressed proteins with picroside biosynthesis in *Picrorhiza kurroa* – A proteomics approach. *Proceedings of the National Conference on "Perspectives & Trends in Plant Sciences and Biotechnology" (PTPB-2014)*, February 21st – 23rd, 2014, Punjab University, Chandigarh.