



# Metabolic signatures provide novel insights to *Picrorhiza kurroa* adaptation along the altitude in Himalayan region

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## Abstract

**Introduction** Along the altitude, environmental conditions vary significantly that might influence plant performance and distribution. Adaptation to these changing conditions is a complex biological process that involves reprogramming of genes, proteins and metabolites. The metabolic response of medicinal plants along the altitude has been less explored yet.

**Objectives** In the present study, we investigated the adaptation strategies of *Picrorhiza kurroa* Royle ex Benth. along the altitude in organ specific manner using metabolomic approach.

**Methods** *Picrorhiza kurroa* plants at flowering stage were randomly sampled from three altitudes viz. 3400, 3800 and 4100 masl in the Himalayan region. Leaf, root and rhizome were used for LC–MS based non-targeted metabolite profiling and targeted analysis of sugars, amino acids, picrosides and their corresponding phenolic acids.

**Results** A total of 220, primary and secondary metabolites (SMs) were identified ( $p < 0.05$ ) representing an extensive inventory of metabolites and their spatial distribution in *P. kurroa*. Differential accumulation of metabolites suggests source–sink carbon partitioning, occurrence of partial TCA cycle, ascorbate metabolism, purine catabolism and salvage route, pyrimidine synthesis, lipid alteration besides gibberellins and cytokinin inhibition might be an adaptive strategy to alpine environmental stress along the altitude. Further, marked differences of organ and altitude specific SMs reflect alteration in secondary metabolic pathways. Significant accumulation of picrosides suggests their probable role in *P. kurroa* adaptation.

**Conclusion** This study provides a platform that would be useful in deciphering the role of metabolites considered to be involved in plant adaptation.

**Keywords** Adaptation · Altitude gradient · Metabolomics · *Picrorhiza kurroa* · Picrosides · Secondary metabolites

## Abbreviations

masl	Meters above sea level
PI	Picroside I
PII	Picroside II
PIII	Picroside III
PIV	Picroside IV
CA	Cinnamic acid
VA	Vanillic acid

FA	Ferulic acid
<i>p</i> -CA	<i>p</i> -Coumaric acid
His	Histidine
Arg	Arginine
Thr	Threonine
Pro	Proline
Met	Methionine
Lys	Lysine
Tyr	Tyrosine
Leu	Leucine
Ile	Isoleucine
Trp	Tryptophan
IC50	50% Inhibition concentration
N	Nitrogen
C	Carbon
SMs	Secondary metabolites
PCA	Principal component analysis
PLS-DA	Partial least squares-discriminant analysis

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## 1 Introduction

Adaptation is an evolutionary process that increases plant survival in a newly encountered climate condition (Endler 1986). Molecular level understanding of adaptation to climate change has an important implication in evolutionary ecology. Altitude offers steep environmental gradients across short spatial distance representing exciting biological experiments in nature (Körner 2007). Along the altitude, environmental factors like low and high-temperature, UV-radiation, hypoxia, wind speed, soil conditions, duration of snow cover and length of vegetation period vary significantly that affect the plant performance and distribution (Körner 2003). For example, it has been reported that an increase of 8% of total irradiance, 9% of UV-radiation and 18% of erythemal effective radiation per 1000 m of altitude (Blumthaler et al. 1997). In addition, mean air temperature also decreases by 6–7 °C per 1000 m of altitude (Woodward 1983). Altitude gradients have been used to explore adaptation mechanism of plants including *Arabidopsis*, however, information regarding medicinal plant adaptation is less available (Zhao et al. 2019; Montesinos-Navarro et al. 2011; Gonzalo-Turpin and Hazard 2009). Medicinal plants are rich source of bioactive phytochemicals that have been used for discovery and development of modern therapeutics. *Picrorhiza kurroa* Royle ex Benth. (Plantaginaceae) is one such high altitude medicinal plant having medicinal values like hepatoprotective, anti-inflammatory, immune-stimulatory and free radical scavenging activity (Kumar et al. 2017). The medicinal importance of *P. kurroa* has been attributed to metabolites especially iridoid glycosides, viz. picrosides. Besides their medicinal importance, it has also adaptive significance which is reflected by its successful acclimation in the Himalayan niche ranges from 3000 to 5000 m above sea level (masl) (Kumar et al. 2017). To maintain their internal homeostasis, plants synthesize and store a variety of metabolites. It is believed that storage and synthesis of metabolites are used by plants for survival in their niche to combat various biotic and abiotic stresses (Weng 2014; Fürstenberg-Hägg et al. 2013). However, less information is available about how metabolic constituents of plant respond to multiple environmental variables along the altitude gradient in the Himalayan region. Therefore, spatial estimation of metabolites provides an insight into different biochemical processes and their interactions with environment.

Study of metabolites will help to unravel plant adaptation strategies through the understanding of complex molecular interactions that occur inside biological system (Fiehn 2002). Metabolomic techniques have been widely adopted to perform metabolite profiling and pathway

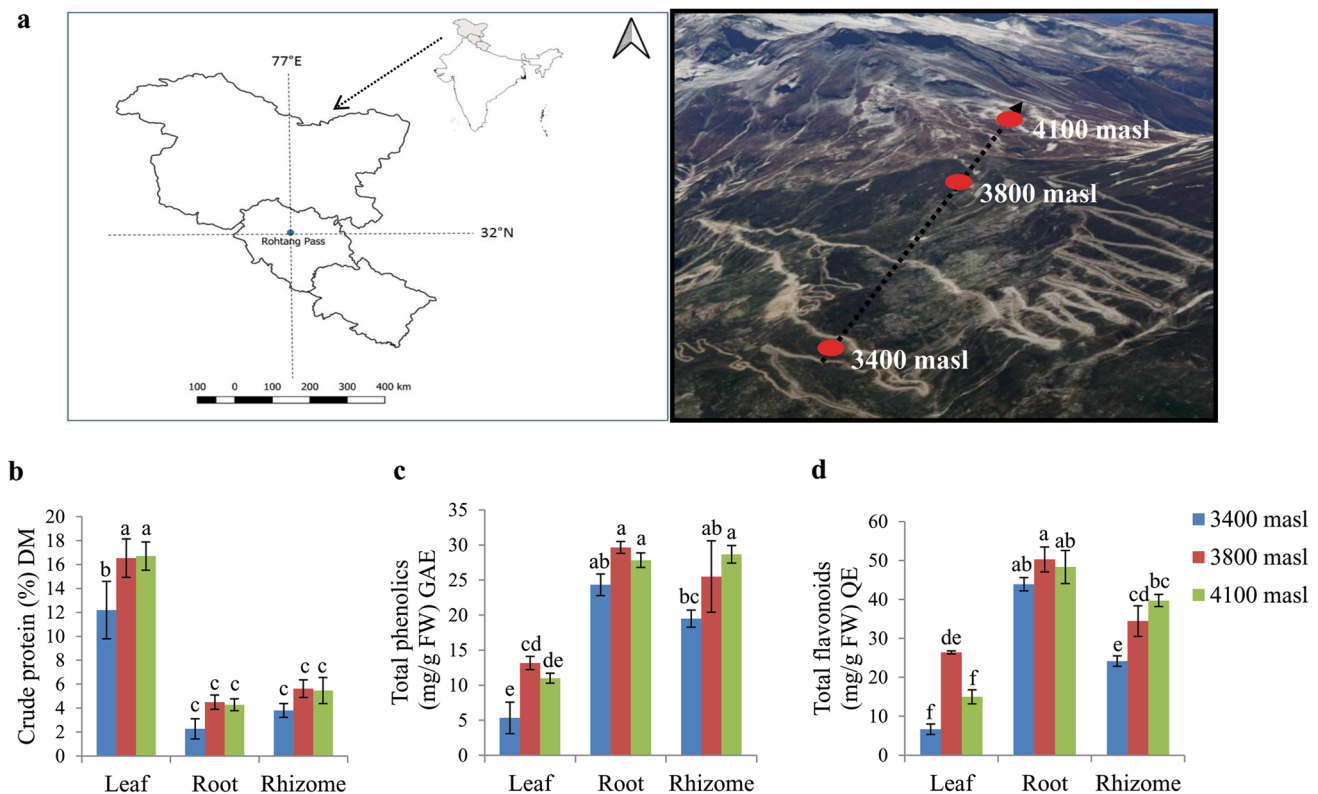
analysis in response to different stress conditions (Luo et al. 2019; Jänkänpää et al. 2012; Urano et al. 2009). Alseekh and Fernie (2018) summarized the metabolic diversity and coverage in past 20 years and mentioned an impressive number of metabolites identified in *E. coli* (700), human (8000) and plants (> 14,000) which are far less than an exact number within *E. coli* (3700), human (114,100) and plants (> 200,000). Several studies have been performed to screen metabolic biomarkers in transgenic/mutant and environmentally challenged plant for better understanding of metabolic pathways and their regulation inside the cell (Hagel et al. 2015; Fiehn et al. 2000). They revealed that plant respond to different environmental factors in a highly coordinated manner results fabrication of numerous primary and secondary metabolites in a tissue-specific manner.

Metabolic constituent of plants varied according to plant genotype, biotic and abiotic conditions (Cohen et al. 2016; Hofmann et al. 2010). Most of the previous metabolic studies were performed under controlled conditions in growth chambers. However, in natural niche, plant encountered more than one environmental condition simultaneously, that results in diverse metabolic composition. Therefore, the metabolomic study of plants growing in natural habitat under tight maintenance of environmental variables illustrates a broader understanding about metabolite functions that play an important role in adaptation. Here, we performed organ and altitude specific non-targeted metabolite profiling and targeted analysis of sugars, amino acids, picrosides (I–IV) and their corresponding phenolic acids. This study will be useful for understanding the role of metabolites which is likely contribute to determine plant phenotype essential for stress adaptation.

## 2 Materials and methods

### 2.1 Chemicals

The standards of picrosides (I–III) and catalpol were purchased from Sigma-Aldrich, India. Picroside IV was procured from SCGLP, China. Methanol, formic acid, acetonitrile were of HPLC grade (Sigma-Aldrich, India). Milli-Q water was obtained from Milli-Q purification system (Millipore, Bedford, MA, USA) for sample and standard preparation. Amino acid mixture, cinnamic acid (CA), vanillic acid (VA), ferulic acid (FA), *p*-coumaric acid (*p*-CA) and o-phthalaldehyde (OPA) were procured from Sigma-Aldrich, India.



**Fig. 1** a Map illustrating the location of *P. kurroa* sampling site in Himalayan region (Qgis software v. 3.0). Organ wise quantitative analyses of biochemical indices along the altitude in terms of b total protein content, c phenolics and d flavonoids. Data are shown as

mean  $\pm$  S.D (n=3) and different letters indicate significant differences ( $p < 0.05$ ) using two-way ANOVA followed by Tukey HSD post-hoc test

## 2.2 Sample collection and extraction

*Picrorhiza kurroa* plants were collected at flowering stage from north facing slope of mountain near Rohtang Pass, Himachal Pradesh, India ( $32^{\circ}22'04''N$ ,  $77^{\circ}15'17''E$ ) for altitude ranging from 3400–4100 masl (Fig. 1a) and authenticated by Biodiversity Division, CSIR-IHBT, Palampur with voucher specimen number PLP 15,384. Three different altitudes namely 3400, 3800 and 4100 masl were selected for sample collection between 10–12 am on clear weather in mid-July 2017 (Fig. S1). The average temperature of 3400, 3800 and 4100 masl were  $19^{\circ}C$ ,  $16^{\circ}C$  and  $8^{\circ}C$ , respectively at the time of harvesting. From 3–4 sites at each altitude, 40–50 plants were selected randomly and frozen in liquid nitrogen instantly. Among them, 10–12 plants were randomly picked for metabolite extraction. Metabolites were extracted from approximately 100 mg of each frozen tissue in 70% methanol (Joshi et al. 2015) and respective pooled samples injected thrice for metabolite profiling. The two factorial experimental design, comprising (i) altitude and (ii) plant organ enable to speculate both altitudinal as well as organ impact on adaptive strategies.

## 2.3 Estimation of total protein, phenolics, flavonoids, iridoid content and antioxidant activities

Total crude protein content was determined from 200 mg dry weight sample using the Kjeldahl method as described previously (Magomya et al. 2014). Total phenolics, flavonoids and antioxidant activities (ABTS and DPPH assays) were determined as described earlier (Joshi et al. 2015). Gallic acid and quercetin standards (Sigma-Aldrich, India) were used for quantification of total phenolics and flavonoids, respectively. Estimation of total iridoid content was performed as described earlier using catalpol as a standard (Narayanan and Akamanchi 2003).

## 2.4 Determination of sugar content

Approximately, 150 mg fresh tissue was extracted in 10 mL deionized water, incubated at  $70^{\circ}C$  for 45 min with intermittent shaking followed by centrifugation for sugar quantification using high performance anion exchange chromatography (HPAEC) as described previously (Dadwal et al. 2018; Monti et al. 2017; Pico et al. 2015). Standards of inositol,

rhamnose, glucose, fructose, sucrose and raffinose (Sigma-Aldrich, India) were used for absolute quantification of sugars in all samples.

## 2.5 Determination of amino acids and phenolic acids

Amino acid analysis was performed as described previously (Agrawal et al. 2019), while quantification was carried out using calibration curves of amino acid mixture. For determination of phenolic acids; CA, VA, FA and *p*-CA, 1  $\mu$ L methanolic extract of each sample was injected in ACQUITY UPLC BEHC18 column (2.1  $\times$  100 mm, 1.7  $\mu$ m particle size) installed on ultra performance liquid chromatography (UPLC) system (Waters, India). Samples were separated using two-solvent system (A; 0.1% trifluoroacetic acid in acetonitrile and B; 0.1% trifluoroacetic acid in water) having gradient program; 0–4 min 10–20% A, 4–6 min 20–40% A, 6–8 min 40–50% A, 8–9 min 50–40% A, 9–10 min 40–20% A, 10–12 min 20–10% A and finally 90% B for next two min with flow rate of 0.3 mL min<sup>-1</sup>. Elution was monitored at 280 nm using a photodiode array detector (PDA) detector and column temperature was maintained at 30 °C. Phenolic acids were quantified using calibration curves of standards on same conditions.

## 2.6 Determination of picrosides content

Analysis of picrosides (I–IV) was performed using hydrophilic interaction chromatography with an Eclipse Plus C18 RRHD column (2.1  $\times$  150 mm, 1.8  $\mu$ m) installed on Agilent 1290 Infinity II UHPLC system (Agilent, Santa Clara, USA). Samples were separated using two-solvent system [A; 0.1% formic acid in water and B; 0.1% formic acid in acetonitrile: methanol (50:50)] having gradient programs; 40–50% B up to 5 min and reduced to 40% B within 8 min with a flow rate of 200  $\mu$ L min<sup>-1</sup>. The injection volume was 2  $\mu$ L. Elution was monitored at 270 nm using a PDA detector and column temperature was maintained at 30 °C. Picrosides (I–IV) were identified by comparing the retention time and mass spectra, while quantified using calibration curves of picroside standards.

## 2.7 Metabolite profiling

Plant organs (leaf, root and rhizome) and altitudes (3400, 3800 and 4100 masl) specific non-targeted metabolite profiling of *P. kurroa* was performed on a high-resolution 6560 Ion Mobility Q-TOF LC/MS (Agilent, Santa Clara, USA). Samples were separated using two-solvent system; A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) having gradient program; 90–50% A for 4 min, 20% A for 9 min, 10% A for 11 min and stopped at 90% A for

19 min with flow rate of 0.22 mL/min. Mass spectrometry parameters were as follow; ionization mode, ESI positive; drying gas temperature, 300 °C; drying gas flow, 5.0 L/min; nebulizer gas pressure, 35 psi; capillary voltage, 3500 V; fragmentor voltage, 400 V; detection window, 100 ppm; MS scan range and rate, 100–3000 *m/z* at 1 spectra/s; MS threshold, 200; MS/MS threshold, 5. Metabolites were extracted from the total ion chromatograms (TICs) based on their *m/z* ratio, retention time and ion intensities using molecular feature extraction (MFE) algorithm. Data was processed using Mass Hunter Qualitative Analysis software (v. B.06.00, Agilent technology) to create .cef files, which were imported into Mass Profiler Professional (MPP) software (v. B.12.00, Agilent) for further data analysis (Wang et al. 2017). The metabolites with absolute abundance greater than 5000 counts were aligned through retention time and accurate mass. The tolerance window of retention time and accurate mass were 0.08 min and 15 ppm, respectively. Alignment, normalization, defining the sample sets and filtering by frequency analysis were applied to process the data. The significant differences among the mass intensities were assessed using ANOVA ( $p < 0.05$ ) followed by Benjamini–Hochberg FDR correction to determine differential metabolites. Compounds of significant differences ( $p < 0.05$ ; log fold change cut off = 1.2) were searched against the METLIN database using “ID browser” for metabolite identification.

## 2.8 Statistical analysis and data visualization

Data was expressed as mean  $\pm$  standard deviation (SD). To analyze significant differences among samples, two-way ANOVA followed by Tukey HSD post-hoc test was performed in R software (v. 3.6.1) with “psych”, “car”, “lsmeans” and “multcomp” packages. Further, multiple linear regression plots among picrosides and corresponding phenolic acids were created with “ggplot2”. Principal component analyses (PCA) was executed with ‘prcomp’ function (“stats” package) and values of variables were scaled before analysis. Partial least squares-discriminant analysis (PLS-DA) was conducted in “pls” package, whereas “corrgram” package was used for correlation plot. Heatmaps were generated using Multi Experiment Viewer (MeV) software (v. 4.9.0).

## 3 Results

### 3.1 Biochemical analysis of leaf, root and rhizome along the altitude

The highest protein content was observed in leaf (12–17%) followed by rhizome (3–6%) and root (2–4%). This organ wise variation also associated with altitude as significant



increase was found at higher altitudes in leaf (Fig. 1b). It also indicated a significant increase in total N content along the altitude in leaf (1.95–2.67%). In contrast to protein content, total phenolics and flavonoids were lesser in leaf (5.33–13.16 and 6.66–26.38 mg/g FW) as compared to root (24.33–29.66 and 43.88–50.27 mg/g FW) and rhizome (19.5–28.66 and 24.16–39.72 mg/g FW). Total phenolics and flavonoid content were increased significantly in rhizome along the altitude, whereas leaf and root showed significant increase of phenolics at higher altitudes, while flavonoids at 3800 masl (Fig. 1c, d). Antioxidant potential was assessed using DPPH and ABTS assays for organ and altitude in terms of IC<sub>50</sub> value. The IC<sub>50</sub> of both assays significantly decreased at higher altitudes in rhizome and leaf but no significant changes were noticed in root among altitudes (Table 1; Fig. S2a, b). Lesser value of IC<sub>50</sub> indicates a relative higher antioxidant potential suggesting an increase in antioxidant activity at higher altitudes. These IC<sub>50</sub> values of DPPH and ABTS showed strong negative correlation with phenolics ( $r = -0.77$  and  $r = -0.86$ ) and flavonoids ( $r = -0.84$  and  $r = -0.89$ ) indicate that increased phenolic and flavonoid content corresponded to decreased IC<sub>50</sub> values (Fig. S2c). Moreover, a strong positive correlation was observed among DPPH and ABTS ( $r = 0.98$ ,  $R^2 = 0.95$ ) and phenolics with flavonoids ( $r = 0.94$ ,  $R^2 = 0.89$ ) showing suitability and reliability of these assays.

### 3.2 Targeted analysis of sugars and amino acids along the altitude

Sugar level in plants has been affected by different stress conditions (Rosa et al. 2009). Figures 2a and S3 displayed the alteration of sugars (glucose, fructose, sucrose, raffinose, rhamnose and inositol) in leaf, root and rhizome along the altitude. In leaf, rhamnose was accumulated along

the altitude, whereas inositol was depleted. Root showed maximum accumulation of rhamnose and raffinose at 3800 masl followed by 4100 masl. However, both rhamnose and raffinose were significantly elevated only at 4100 masl in rhizome. Glucose was significantly increased at 4100 masl in both root and rhizome, while sucrose (in leaf, root and rhizome) and raffinose (in rhizome) displayed a biphasic pattern of accumulation; decreased at 3800 masl but increased at 4100 masl compared with 3400 masl.

Further, amino acid content was also altered along the altitude in organ specific manner (Figs. 2b and S4). In leaf, histidine (His) and arginine (Arg) were significantly elevated at 3800 and 4100 masl, whereas proline (Pro) was increased only at 4100 masl. Low abundant amino acids like methionine (Met) and tryptophan (Trp) significantly increased along the altitude, whereas His and Arg profound at 3800 masl followed by 4100 masl in root. In rhizome, Trp accumulated significantly along the altitude, while Arg increased at 3800 masl and thereafter, no significant change was noticed at 4100 masl. Tyrosine (Tyr) and Met displayed a biphasic pattern of accumulation along the altitude in rhizome; decreased at 3800 masl but increased at 4100 masl.

PCA of sugars and amino acids revealed 66.3% of total variance in whole dataset which is explained by two principal components (PCA1 and PCA2; Fig. 2c). PCA1 accounted for 41.5% of variance, which was mainly influenced by sucrose, inositol, Met, Trp and Pro. Lesser angle between vectors (e.g. Met, Trp and sucrose) showed a positive correlation. Likewise, PCA2 contributed 24.8% of variance in which major variables are raffinose, glucose, rhamnose, fructose, His, Arg, Lys, Leu and Thr. Arg-His, Ile-Tyr and glucose-rhamnose also revealed positive correlations. The biplot also showed that raffinose, rhamnose, glucose and Arg mainly accumulated in root at higher altitudes. Sucrose, Met, Trp, Ile and Tyr showed higher levels in rhizome, while inositol and fructose mainly accumulated in leaf.

### 3.3 Targeted analysis of picrosides and corresponding phenolic acids along the altitude

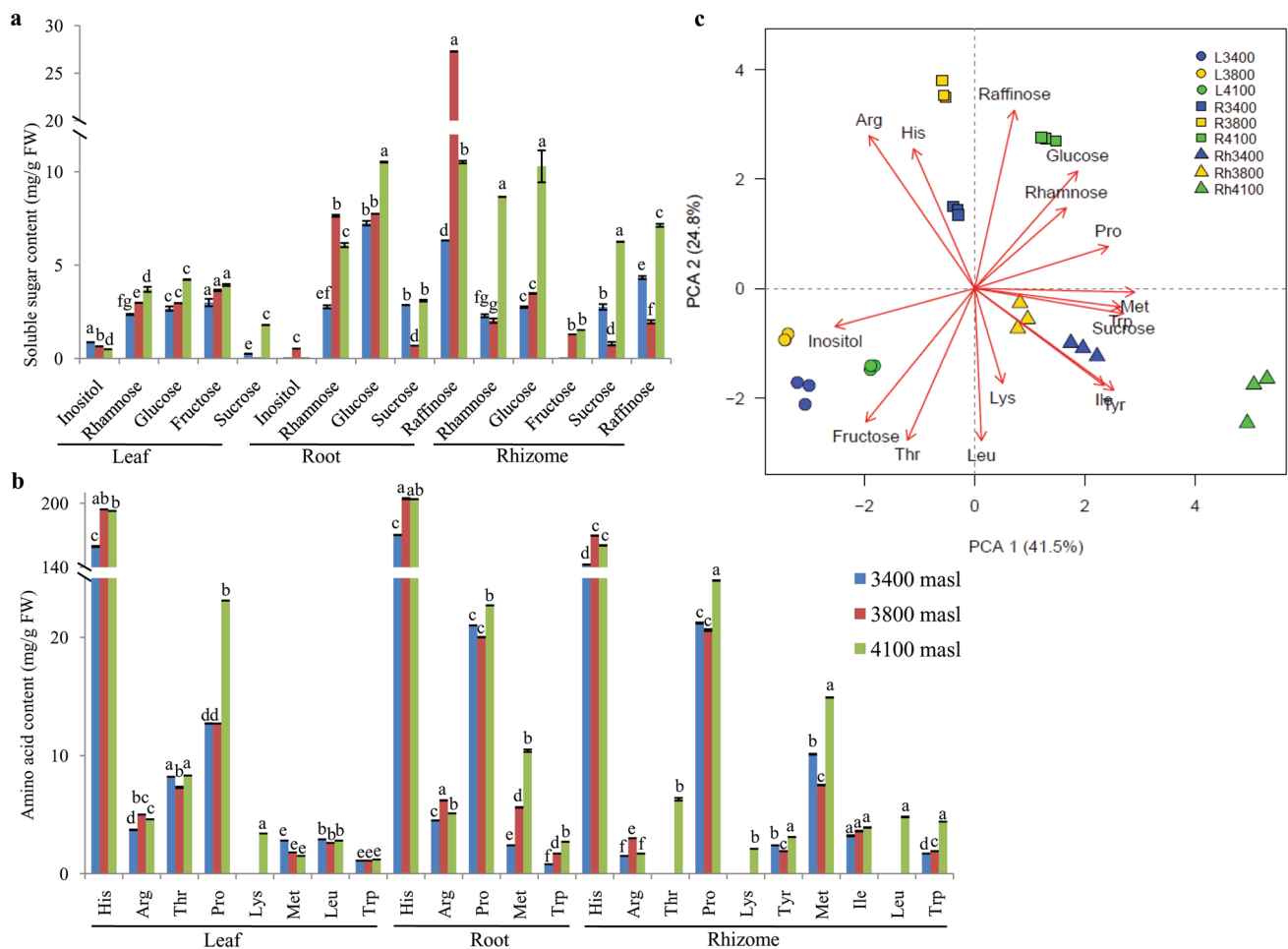
The picrosides (PI, PII, PIII and PIV) were analyzed using UHPLC (Fig. S5). The content of PI was higher in leaf compared to rhizome and root at 3800 and 4100 masl, however at 3400 masl, rhizome showed the highest accumulation (Fig. 3a). The level of PII was higher in root compared to rhizome and leaf at 3800 and 4100 masl, while at 3400 masl, rhizome showed maximum content (Fig. 3b). Leaf and rhizome showed opposite trends for PIII as increasing and decreasing pattern observed in leaf and rhizome along the altitude, respectively; however, no significant changes were noticed in root (Fig. 3c). Interestingly, PIV showed a mixed pattern of accumulation in root and rhizome. Compared

**Table 1** Organ and altitude specific antioxidant potential in terms of DPPH and ABTS assays represented as IC<sub>50</sub> value (μg/μl FW)

Sample	DPPH IC <sub>50</sub> (μg/μl FW)	ABTS IC <sub>50</sub> (μg/μl FW)
L3400	155.85 ± 8.48 <sup>a</sup>	116.20 ± 11.38 <sup>a</sup>
L3800	60.98 ± 1.08 <sup>bc</sup>	56.35 ± 2.20 <sup>b</sup>
L4100	72.41 ± 5.18 <sup>bc</sup>	62.35 ± 4.03 <sup>b</sup>
R3400	46.58 ± 5.51 <sup>c</sup>	34.99 ± 6.18 <sup>b</sup>
R3800	43.62 ± 4.90 <sup>c</sup>	31.87 ± 7.67 <sup>b</sup>
R4100	46.29 ± 5.19 <sup>c</sup>	35.68 ± 6.68 <sup>b</sup>
Rh3400	86.03 ± 3.87 <sup>b</sup>	56.28 ± 2.98 <sup>b</sup>
Rh3800	66.28 ± 3.42 <sup>bc</sup>	47.11 ± 3.23 <sup>b</sup>
Rh4100	61.74 ± 2.82 <sup>bc</sup>	45.40 ± 0.77 <sup>b</sup>

L leaf, R root, Rh rhizome

Different letters indicate significant differences ( $p < 0.05$ ) using two-way ANOVA followed by Tukey HSD post-hoc test



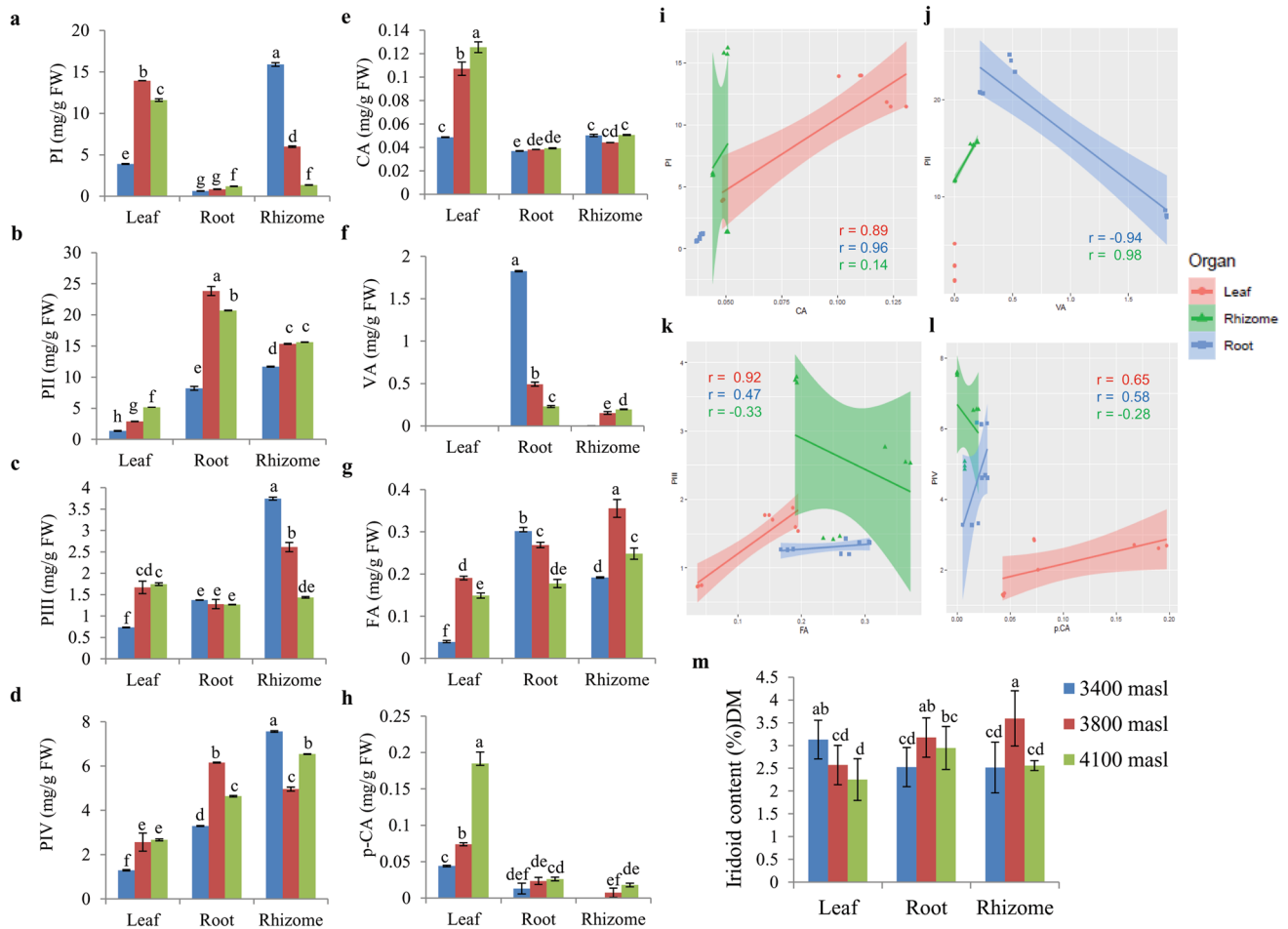
**Fig. 2** Determination of primary metabolites in *P. kurroa* by HPAEC and UPLC; **a** Soluble sugars, **b** amino acids and **c** PCA biplot where individuals colored by altitude, shaped by organ and arrows showing loadings of sugars and amino acids. *L* leaf, *R* root and *Rh* rhizome.

Significance was examined by two-way ANOVA followed by Tukey HSD post-hoc test ( $p < 0.05$ ) for each variable (sugar/amino acid) autonomously and shown by different letters

to 3400 masl, PIV was increased in leaf and root, while decreased in rhizome at higher altitude (Fig. 3d). Therefore, distribution and relative content of picrosides are thought to be regulated by environmental conditions is both organ and altitude specific.

To determine whether variation in picroside content among altitude and organ is due to biosynthetic processes, we analyzed CA, VA, FA and *p*-CA; the immediate precursors of PI, PII, PIII and PIV biosynthesis, respectively from the same methanolic extract using UPLC (Figs. 3e–h and S6). CA, responsible for PI biosynthesis showed maximum content in leaf compared to root and rhizome, while increased significantly along the altitude. Regression analysis indicated a strong positive correlation among CA and PI in leaf ( $r = 0.89$ ,  $R^2 = 0.80$ ) and root ( $r = 0.95$ ,  $R^2 = 0.91$ ); however, no significant correlation was observed in rhizome (Fig. 3i). VA thought to be esterified with catalpol to form PII that was not detected in leaf but showed decreasing and

increasing trends along the altitude in root and rhizome, respectively. Likewise, regression analysis also revealed opposite patterns of correlation among VA and PII; as strong positive in rhizome ( $r = 0.98$ ,  $R^2 = 0.96$ ) and strong negative in root ( $r = -0.94$ ,  $R^2 = 0.89$ ; Fig. 3j). The complete biosynthetic route for PIII and PIV has been unexplored yet. Earlier, it was proposed that PIII and PIV are also catalpol derivatives with slight modification in  $R_3$  group with FA and *p*-CA, respectively (Kumar et al. 2013). FA decreased significantly in root along the altitude but leaf and rhizome showed higher accumulation at 3800 masl followed by 4100 masl and 3400 masl, respectively. On other hand, a higher level of *p*-CA was found in leaf compared to root and rhizome besides an increasing trend of accumulation along the altitude. Correlation among FA and PIII showed strong positive in leaf ( $r = 0.92$ ,  $R^2 = 0.84$ ), mild positive in root ( $r = 0.47$ ,  $R^2 = 0.21$ ) and weak negative in rhizome (Fig. 3k). Similarly, regression analysis among *p*-CA and



**Fig. 3** Organ and altitude specific analyses of picosides, phenolic acids and total iridoid content. Relative content of **a** PI, **b** PII, **c** PIII, **d** PIV, **e** cinnamic acid (CA), **f** vanillic acid (VA), **g** ferulic acid (FA), **h** *p*-coumaric acid (*p*-CA) and **m** total iridoids. Data are

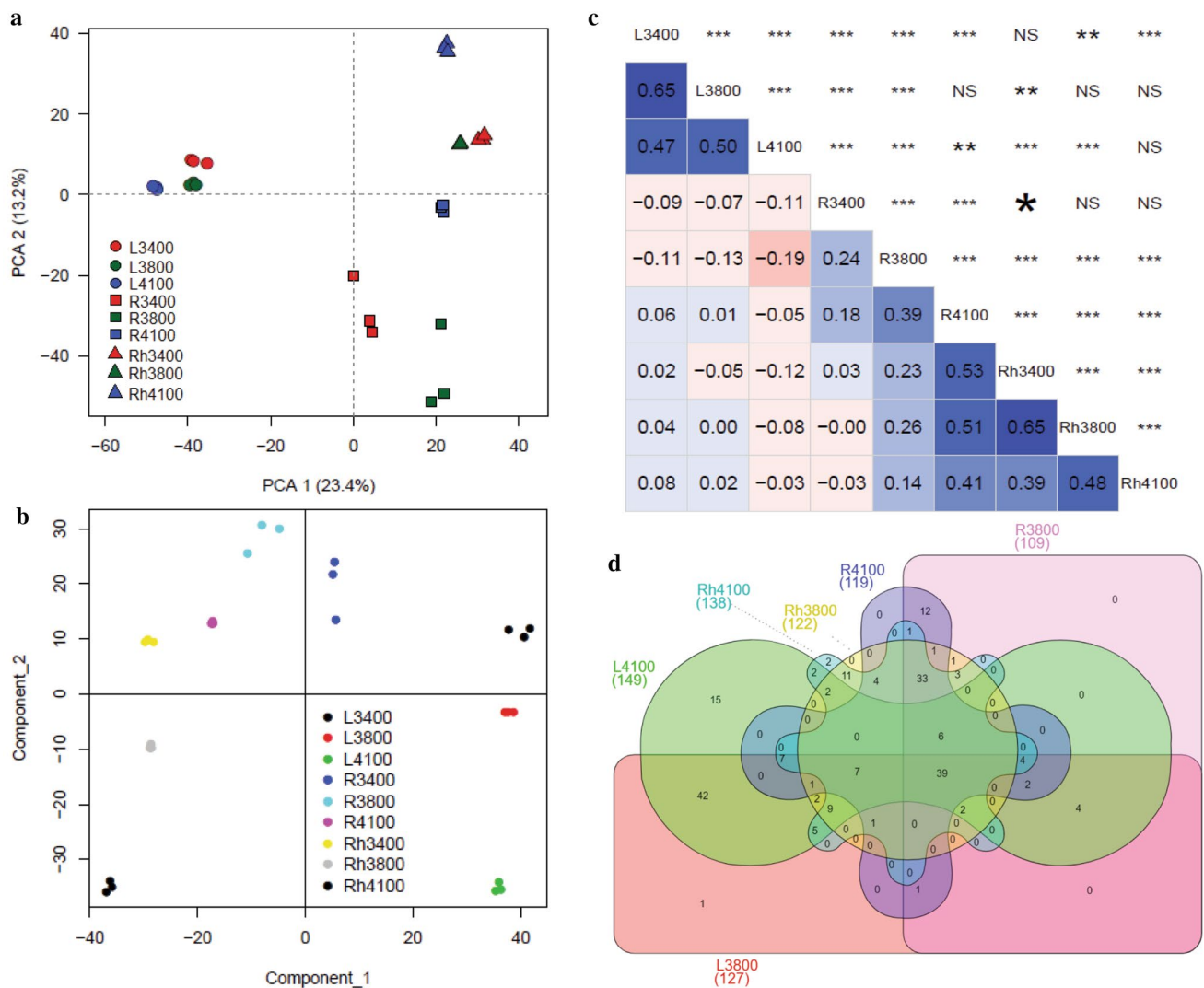
shown as mean  $\pm$  S.D ( $n=3$ ) and significance by different letters evaluated using two-way ANOVA followed by Tukey HSD post-hoc test ( $p < 0.05$ ). Scatter plots depicting correlation coefficient ( $r$ ) among **i** PI and CA, **j** PII and VA, **k** PIII and FA and **l** PIV and *p*-CA

PIV revealed positive correlation in leaf ( $r=0.65$ ,  $R^2=0.43$ ), root ( $r=0.58$ ,  $R^2=0.33$ ) and weak negative correlation in rhizome (Fig. 3l). Furthermore, colorimetric estimation of total iridoid content was also showed significant variation along the altitude in organ specific manner (Fig. 3m). Total iridoid content was decreased significantly along the altitude in leaf, whereas root and rhizome showed maximum content at 3800 masl followed by 4100 masl and 3400 masl, respectively.

### 3.4 Non-targeted metabolite profiling of *P. kurroa* along the altitude

Based on literatures, it is found that leaf, root and rhizome were actively involved in metabolite synthesis and storage which reflects the significance of organ specific study (Campbell et al. 2007). We screened 196 plant metabolites across the altitude gradient in three

different organs ( $p < 0.05$ , Table S1). In order to visualize the metabolic variance among organs and altitude, PCA was performed (Fig. 4a). PCA1 and PCA2 together explained 36.6% of total variance in whole normalized dataset. PCA1 contributed for 23.4% of variance, which separates leaf from root and rhizome, while PCA2 separating root and rhizome with 13.2% of variance. The plot showed much similar metabolic profile among altitudes as compared to organs i.e. leaf, root and rhizome which were dissimilar in metabolic profiling. These metabolic differences were reproducible with three replicates. PCA is an unsupervised dimension reduction technique which focusing on differences between samples and may not within samples groups (Goodacre et al. 2007). Therefore, PLS-DA; one of the supervised data reduction technique was performed on normalized dataset, which highlighted the differences among altitudes and organs more clearly as compared to PCA (Fig. 4b). The result was further



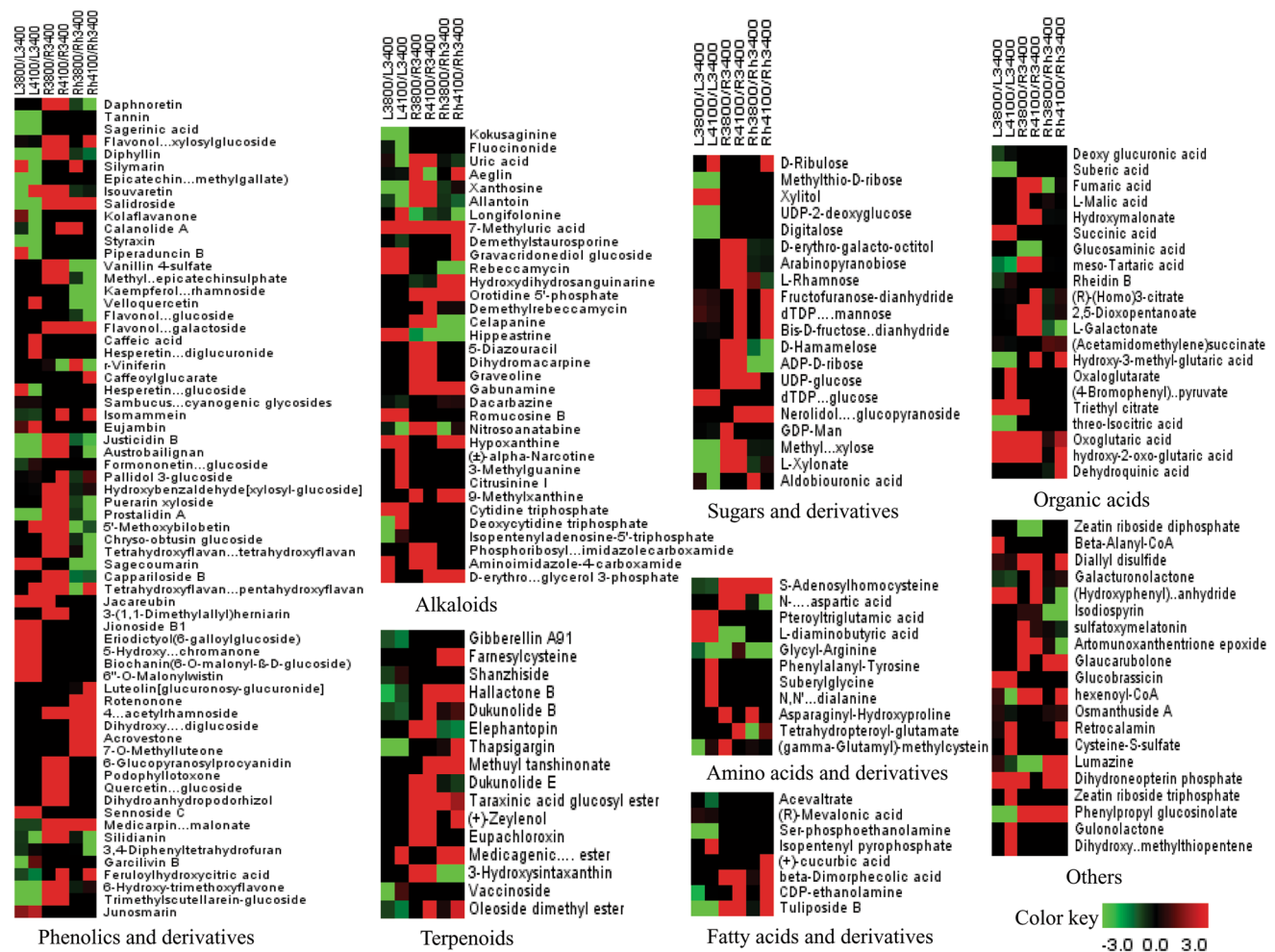
**Fig. 4** Visualization of metabolic differences among altitude and organs in *P. kurroa*. Multivariate analyses are shown as **a** principal component analysis (PCA) and **b** partial least squares discriminant analysis (PLS-DA). **c** Correlogram showing Pearson's correlation coefficient ( $r$ ) in lower panel and asterisks represent associated  $p$

validated through correlation study visualized by correlation matrix (Fig. 4c). Both positive and negative correlations were found for three types of organs. The pair wise comparison of different organs along the altitude resulted in Pearson correlation value ranged from 0.65–0.47 for leaf, 0.39–0.18 for root and 0.65–0.39 for rhizome. These correlations are positive; however, following decreasing trend along the altitude suggesting the same organ across altitude differ in their metabolic constituents. Among different organs, leaf showed a negative correlation with root, root and rhizome showed positive correlation, while no significant correlation was noticed among leaf and rhizome. Moreover, organ-specific comparison of differentially accumulated metabolites normalized to 3400 masl

value ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ) in upper panel. **d** Venn diagram depicting organ-specific comparison of differentially abundant metabolites normalized to 3400 masl using InteractiVenn (<https://www.interactivenn.net>). *L* leaf, *R* root and *Rh* rhizome

in *P. kurroa* along the altitude was performed (Fig. 4d). Among 220 metabolites (196 from non-targeted and 24 from targeted analysis), 39 were common in all the organs. Whereas, root-rhizome shared maximum 33 metabolites, while only nine and two metabolites shared by leaf-rhizome and leaf-root, respectively. It signifies quite dissimilar metabolite profile in leaf from root and rhizome. The analysis also revealed forty-two, twelve and eleven unique metabolites in leaf, root and rhizome, respectively. Together, these results indicated the consistent differences in metabolite composition of *P. kurroa* along the altitude in organ specific manner demonstrating a significant influence of metabolites in adaptation against alpine environmental constraints. Further, identified metabolites are





**Fig. 5** Heat maps showing organ-wise  $\log_2$  fold changes in metabolite levels compared to 3400 masl. Metabolites were arranged into eight classes according to their biochemical groups

classified according to their biochemical groups namely, phenolics and their derivatives, alkaloids, terpenoids, sugars, amino acids, fatty acids and their derivatives, organic acids and others. The abundance of identified metabolites was visualized using heat maps illustrating metabolic diversity (Fig. 5).

## 4 Discussion

Targeted and non-targeted metabolite profiling could contribute effectively to the study of plant stress responses through qualitative and quantitative analysis of compounds which are byproducts of stress metabolism. Here, we performed organ and altitude specific metabolite profiling of *P. kurroa* grown in natural habitat. A clear metabolic difference was observed among organ and altitude in PCA and PLS-DA analysis. This metabolic variation may

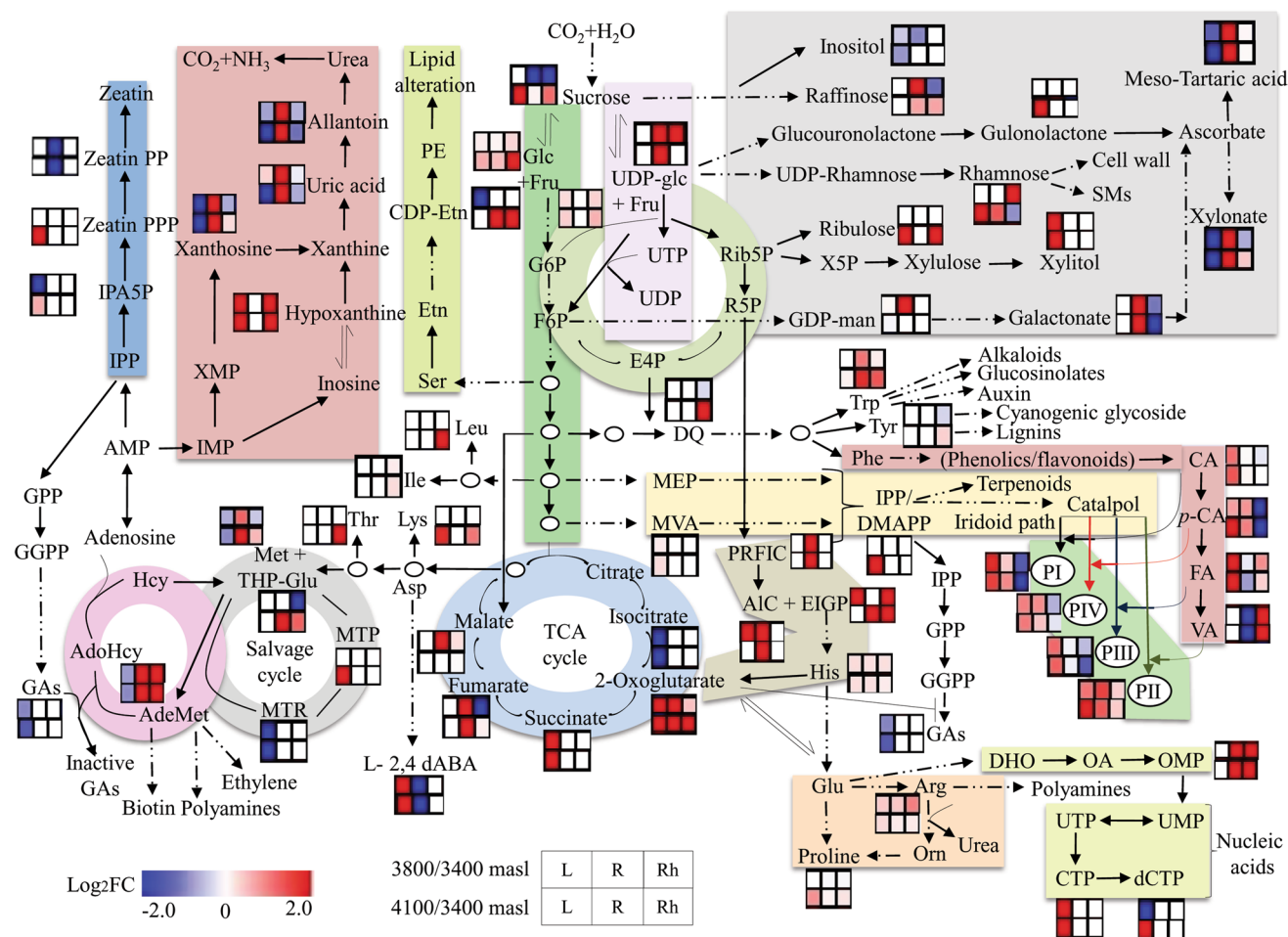
contribute *P. kurroa* tolerance to alpine stress conditions along the altitude.

### 4.1 Alterations of carbon metabolism may provide tolerance to environmental stress

Alteration in primary metabolism is more pronounced to maintain internal homeostasis in response to environmental fluctuations. Sugars have been reviewed as a positive regulator of plant adaptation in combination of multiple environmental stresses (Wulff-Zottele et al. 2010; Rizhsky et al. 2004). Moreover, the increased rate of photosynthesis that occurs along the altitude should lead to higher sugar level; an elevated sugar level along the altitude was reported in *Potentilla saundersiana* (Ma et al. 2015). The accumulation of sugars in this study (Fig. 2a) is in accordance with previous reports demonstrated that the pattern of sugar accumulation is organ and stress-specific (Gill et al. 2001; Morsy et al. 2007). These compatible solutes have been thought to be

involved in plant tolerance by act as both osmolyte and stabilizer of protein and membrane (Rosa et al. 2009). Of particular interest might be the higher accumulation of sucrose in sink tissues (root and rhizome) compared to source (leaf) tissue with altitude suggesting source–sink carbon partitioning at whole-plant level is an adaptive strategy to enhance stress tolerance. Higher accumulation of sucrose at 4100 masl suggests that it act as a major osmoprotectant in *P. kurroa*, whereas decreased level of sucrose and increased level of raffinose at 3800 masl indicates biosynthesis of raffinose from sucrose. Raffinose is a trisaccharide synthesized by stepwise addition of galactose units to sucrose (Chibbar and Båga 2003) and stored in sink tissues. Raffinose acts as an osmoprotectant and antioxidant to counteract the ROS under stress conditions (EISayed et al. 2014). Increased sugars like glucose at 4100 masl might be acting as a respiratory substrate or an osmolyte to maintain cellular homeostasis, while fructose seems to be involved in SM synthesis through the generation of erythrose-4-P; a substrate of shikimate

pathway (Hilal et al. 2004). Further, a significant increase of UDP-glucose level in root and rhizome at higher altitude suggests modulation of carbon metabolism through sucrose degradation by sucrose synthase which favored under hypoxia condition in sink tissues to maintain energy status in cell (Fig. 6). Earlier *M. cajuputi* roots have also showed an increase in the activity of sucrose synthase under hypoxia treatment (Kogawara et al. 2014). A study reported that invertase repression (sucrose degradation to glucose and fructose) and sucrose synthase induction (sucrose degradation to UDP-glc and fructose) conserve oxygen by less costly route (Bologa et al. 2003). The elevated UDP-glc was correlated with the levels of L-rhamnose and salidroside in root and rhizome which supposed to be involved in their synthesis (Fig. 6). L-Rhamnose is a known constituent of primary cell wall, synthesized from UDP-glc by three step process. The previous report has also shown the involvement of rhamnose not only in cell wall biosynthesis but also for synthesis of SMs (Kim et al. 2013). Consistent with this, we



**Fig. 6** Pathways view of metabolite distribution within *P. kurroa* along the altitude. Organ wise log<sub>2</sub> fold changes ratios to 3400 masl are shown in heat maps. Different shades indicate different metabolic

pathways, while single solid line and dashed lines represent single and multiple steps, respectively. Abbreviations of metabolites are given in Table S2

also observed significant up-regulation of phenolic glycoside rhamnose derivative in root and rhizome along the altitude. In addition, UDP-glc also led to higher level of xylose in root which is a constituent of pectic polysaccharide in the cell wall, suggesting remodeling of root cell wall at higher altitudes. Increased level of GDP-mann in root at 3800 masl correlated with galactonate which may lead to ascorbate synthesis (Conklin et al. 1999), while accumulation of gulonolactone in leaf at 4100 masl suggested the possibility of another route for ascorbate synthesis (Valpuesta and Botella 2004). Ascorbate is a well known water soluble antioxidant which regulates various physiological processes including stress tolerance (Smirnov and Wheeler 2000). Despite the identification of ascorbate biosynthetic intermediates, we are unable to detect ascorbate in our study might suggesting metabolization of ascorbate to meso-tartaric acid through C4/5 cleavage, which also elevated significantly in root, while reduced in leaf (Fig. 6). An earlier study also demonstrated that levels of ascorbate and tartrate were positively correlated with light intensity and water stress in green berries (Melino et al. 2011; DeBolt et al. 2008). Thus, differential accumulation of sugars in *P. kurroa* suggests the existence of different regulatory mechanism having an adaptive role at higher altitudes.

#### 4.2 Partial TCA cycle and histidine metabolism could be a strategy of plant adaptation

Significant accumulation of succinic acid in leaf with increasing altitude is consistent with previous reports that succinate accumulates to low oxygen availability in soybean (António et al. 2016) and rice coleoptiles (Narsai et al. 2009). Earlier study reported inhibition of TCA cycle under low O<sub>2</sub> availability and accumulation of succinate by alternative pathways i.e. GABA shunt and alanine metabolism (Rocha et al. 2010). Later on, António et al. (2016) contradicted the previous reports and suggested the existence of TCA cycle along with alternative pathways in hypoxia condition. Under hypoxia, activation of fermentation can lead to lactate and ethanol production while, under simultaneous low phosphorus and O<sub>2</sub> availability, phosphoenolpyruvate fluxed to malate production (Colebatch et al. 2004). Malate accumulation in root and rhizome at 3800 masl might be due to carboxylation of phosphoenolpyruvate so that additional ATP was generated. Malate may also be metabolized to succinate through fumarate (Hochachka 1986) and hence, elevated levels of fumarate were observed in root. Increased level of fumarate was only reported during anoxic germination in rice (Narsai et al. 2009). Therefore, we can suggest the occurrence of partial TCA cycle at higher altitudes in *P. kurroa* likely be an adaptive strategy to multiple constraints (Fig. 6). Further, 2-oxoglutarate; a key regulator of carbon and nitrogen metabolism was significantly increased in all

organs along the altitude which might provide carbon source to amino acids and SMs biosynthesis (Noguchi and Yoshida 2008; Kliebenstein et al. 2001). Histidine degradation synthesizes 2-oxoglutarate during oxidative stress by blocking citrate decomposition, which act as an effective ROS scavenger by decreasing pro-oxidant NADH production through diversion from TCA cycle (Lemire et al. 2010). However, significant accumulation of histidine in all organ at higher altitudes might indicate anabolism (Fig. 6) from ribose phosphate (R5P) through 1-(5'-phosphoribosyl)-5-formamido-4-imidazolecarboxamide (PRFIC), 5-aminoimidazole-4-carboxamide (AIC) and D-erythro-1-(imidazol-4-yl) glycerol 3-phosphate (EIGP). Under heavy metal stress, histidine is involved in plant tolerance by act as a metal chelator that has low metabolic cost as compared to others (Bing et al. 2016; Zemanová et al. 2014; Zechmeister 1995). Thus, His levels maintained by balance between its degradation and synthesis could be another adaptive strategy profound at high altitudes.

#### 4.3 Modulation of amino acid metabolism predominantly takes place at high altitude

Amino acids were involved in nitrogen metabolism, signaling and plant stress responses besides being as a protein constituent (Hildebrandt et al. 2015). It is believed that accumulation of amino acids under stress might be associated with nitrogen storage and act as a precursor for synthesis of SMs. Considerable reports have been available addressing amino acid accumulation under abiotic stresses (Batista-Silva et al. 2019; Hildebrandt 2018). An earlier report demonstrated that low temperature increases protein content in plants, thereby, decrease freezing point to prevent ice formation (Guy 1990). Consistent with this, we also found an enrichment of mineral nutrient particularly, N and protein content along the altitude (Fig. 1b), which could explain the increased accumulation of amino acids in *P. kurroa* along the altitude. Therefore, increased synthesis and storage of amino acids might be one of the adaptive responses to alpine stress. Among various amino acids, Arg has highest N to C ratio that makes it possible storage form of organic nitrogen (Winter et al. 2015). Accumulation of Arg at higher altitude can serve as precursor for various SMs especially nitrogenous compounds like NO and polyamines which play an important role in abiotic stress tolerance (Domingos et al. 2015; Alcázar et al. 2010). However, a slight reduction in Arg content from 3800 to 4100 masl might be correlated with Pro accumulation at 4100 masl (Fig. 6). Proline, a proteinogenic amino acid is often documented as a stress-induced osmoprotectant, ROS scavenger, DNA, protein and membrane stabilizer (Krasensky and Jonak 2012) has been reported in response to drought (Choudhary et al. 2005), salinity (Yoshida et al. 1995), UV irradiation (Saradhi et al.

1995), heavy metals (Schat et al. 1997), oxidative and biotic stress (Yang et al. 2009; Fabro et al. 2004). Usually, Pro synthesized from glutamate; however, it may follow alternative pathway viz. ornithine pathway in which Arg degraded to ornithine followed by transamination along with 2-oxoglutarate to P5C and glutamate, subsequently converted to proline (Szabados and Savoure 2010). Arg catabolism for Pro synthesis releases  $\text{NH}_3$  and could serve to N mobilization as a defense strategy at 4100 masl. Modulation in Met metabolism can't be ignored as higher levels of S-adenosylhomocysteine (AdoHcy) along with aspartate derivative were observed in root and rhizome. Therefore, aspartate might serve as a donor of carbon and amino skeleton to AdoHcy leading to increased Met and tetrahydropteroyltri-L-glutamate (THP-Glu). This may lead to further synthesis of ethylene, polyamines or various SMs (Hacham et al. 2017). Methionine is a well-known ROS scavenger, which may provide oxidative stress resistance via pentose phosphate pathway (Campbell et al. 2016) being abundance of various pentose sugars (ribulose, xylose) were observed at high altitude. However, decreased Met content in leaf can be correlated with decreased synthesis and reduced salvage cycle through AdoHcy and methylthioribose (MTR). Significant increase in Trp content in both root and rhizome with increasing altitude could explain the higher level of SMs such as glucosinolates (Glucobrassicin, 3-phenylpropyl glucosinolate). Moreover, increased Trp level also reduces Cd availability to plants through interaction of metal ions with bivalent Trp side chain (Li and Yang 2003) in addition to cold tolerance (Hildebrandt 2018). Therefore, organ and altitude specific accumulation of amino acids likely reflects their role in alpine stress tolerance.

#### 4.4 Altitude exerts influence on nucleotide metabolism in organ specific manner

We observed purine catabolism in root of *P. kurroa* (Fig. 6). Allantoin; a stress related purine metabolite and uric acid were accumulated in root with increasing altitude, suggesting nitrogen recycling and remobilization initiated from xanthine oxidation through allantoin hydrolysis to release ammonia (Watanabe et al. 2014). However, leaf and rhizome showed elevated levels of hypoxanthine through inosine degradation. In contrast to root, where purine catabolism was predominating along the altitude, salvage pathway of purine nucleotides might be operated in leaf and rhizome to adjust nucleotide pool. Compared to de novo synthesis, salvage pathway is less energy consuming route to reuse nucleotides. This reveals organ specific adaptive strategies consistent with previous findings such as drought (Yobi et al. 2013), salinity (Suzuki-Yamamoto et al. 2006) and low nutrient availability (Coneva et al. 2014). In contrast to purine degradation, pyrimidine nucleotide synthesis

induced either by *denovo* or salvage pathway as increased levels of CTP and dCTP were found in leaf along the altitude (Fig. 6). Also, accumulation of orotidin monophosphate (OMP); an intermediate precursor of pyrimidine nucleotides was noticed in root and rhizome suggesting the existence of *denovo* pathway for their synthesis (Suzuki-Yamamoto et al. 2006). These pyrimidine nucleotides could be used for carbohydrate metabolism such as synthesis of nucleotide sugars and transport of sucrose. A higher level of CDP-ethanolamine (CDP-Etn) in root and rhizome at 4100 masl may lead to phosphatidyl-ethanolamine (Sahu and Shaw 2009) suggesting an alteration in lipid composition. Previous reports on sugar bean roots and *B. napus* revealed that lowering of phosphatidylcholine (PC)/phosphatidylethanolamine (PE) ratio (Lindberg et al. 2005) and enrichment of phosphatidylethanolamine (Tasseva et al. 2004) improved freezing tolerance. Therefore, nucleotide metabolism highlighted the differences among organs and suggesting purine catabolism in root serve N that might be transported to leaf, which becomes more evident with increasing altitude.

#### 4.5 Altitude gradient is associated with extensive change in secondary metabolites

In addition to variation in primary metabolites, marked differences of organ and altitude specific SMs were noticed, which reflect the alteration of secondary metabolic pathways. Unlike primary metabolites, SMs restricted to particular species or families (Pichersky and Gang 2000). Also, plant habitat makes a profound effect on the quality and quantity of SMs production (Endress 1994). We found an increased accumulation of SMs particularly, phenolics and flavonoids in organ and altitude specific manner (Fig. 1c, d) which might be due to allocation of increased carbon. Phenolics are known to play a key role in flower pigmentation, scavenging of ROS, protection from UV stress, tolerance toward mineral nutrient deficiency and defense against pathogens (Winkel-Shirley 2001; Zhishen et al. 1999; Ylstra et al. 1992). In addition, they have been reported to possess anti-inflammatory, antimicrobial and anti-carcinogenic activities, which seem to be beneficial for human health (Butelli et al. 2008). Moreover, reports correlated with accumulation of phenolics to environmental variables in natural condition is not well known; however, a study conducted on several plant species from a gypsum zone reported a positive correlation between accumulation of phenolics/flavonoids and degree of environmental stress (Boscaiu et al. 2010). A significant accumulation of salidroside was noticed in root and rhizome along the altitude (Table S1) which is consistent with previous study that demonstrated high production of salidroside associated with high soil organic matter, low soil pH, high soil N level and low bioavailability of soil P and K (Yan et al. 2004). This suggests the existence of multiple



abiotic factors regulating the salidroside metabolism that might be important for quenching ROS. Oxidative stress, considered to be gateway of most abiotic stresses results due to excessive production of ROS leading to damage of cellular components like DNA, proteins and lipids. Synthesis of eujambin, pallidol-3-glucoside and sagecoumarin might play a crucial role in scavenging free radicals that helps in maintaining cellular redox balance suggesting an adaptive response (Lopresti 2017; Asam Raza et al. 2015; Xu et al. 2014). Interestingly, an epimer of *Sambucus nigra* degraded cyanogenic glycosides increased in leaf with increasing altitude (Table S1) suggesting herbivore defense response through cyanogenesis by releasing cyanide (Gleadow and Woodrow 2000). Significant variation in cyanogenic glycoside would be due to allocation of leaf N and alteration of light was reported in *Eucalyptus* (Burns et al. 2002). Graveoline; a phytotoxic alkaloid earlier reported from *Ruta graveolens* reduces plant height and chlorophyll content; possess antifungal and anticancer activity considered to be involved in allelopathy for their survival (Hale et al. 2004). Allelopathy is a common biological process where one plant inhibits the growth of others through releasing allelochemicals, which act as a means of survival in nature by reducing competition from nearby plants. We found significant accumulation of allelochemicals; graveoline (in roots) and diallylsulphide (in all organs) along the altitude (Table S1) indicates an adaptive response to reduce competition in nutrient deprived condition.

Furthermore, isopentenyl pyrophosphate (IPP) synthesizes either from MVA or MEP pathway acts as a precursor for various terpenoids and endogenous growth regulators. In contrast to significant accumulation of precursor in leaf at higher altitude, reduction of gibberellin (GAs) content was observed in leaf along the altitude suggesting lower level of GAs restricts the plant growth in alpine conditions (Magome et al. 2008; Achard et al. 2008) and hence, provides tolerance to plants. The reduced GAs level at higher altitudes might be due to elevated 2-oxoglutarate dependent biosynthetic inhibition (Griggs et al. 1991) or inactivation by methylation during the conversion of AdeMet to AdeHcy (Fig. 6). Likewise, a significant reduction in *trans*-zeatin riboside diphosphate (zeatinPP) in root along the altitude could reduce *trans*-zeatin production i.e. a kind of cytokinin. While, higher level of various intermediates such as isopentenyladenosine-5'-triphosphate (IPA5P) and *trans*-zeatin riboside triphosphate (zeatinPPP) in leaf at 4100 masl does not lead to zeatin biosynthesis suggesting suppression of biosynthetic pathway in leaf rather than catabolism (Fig. 6). Reduction in endogenous cytokinin associated with growth suppression in stress response is well established (Prerostova et al. 2018; Rivero et al. 2007). Therefore, we hypothesized that hormonal crosstalk might also contribute to *P. kurroa* adaptation along the altitude.

#### 4.6 Organ and altitude specific picrosides accumulation suggests their probable role in *P. kurroa* adaptation

Significant changes in picroside (I–IV) content were observed along the altitude in organ specific manner; however, the role of picrosides in *P. kurroa* adaptation is largely unknown yet. Differential accumulation of picrosides in organs along the altitude might be hypothesized their role in plant adaptation. However, further experiments will be required to validate this hypothesis. Although, the metabolic pathway for picroside biosynthesis is extremely complex and thought to be interconnected through mevalonate (MVA), non-mevalonate (MEP), shikimate/phenylpropanoid and iridoid pathways. Both MVA and MEP pathway contribute isoprene units leading to catalpol formation through iridoid pathway and in final step picrosides I, II, III and IV were synthesized through esterification of catalpol with corresponding phenolic acid: CA, VA, FA and *p*-CA, respectively (Kumar et al. 2013). We found an accumulation of mevalonic acid (MVA) i.e. MVA pathway intermediate and CTP that act as a substrate in MEP route leads to increased IPP content at higher altitude in leaf. In addition, CA, FA and *p*-CA also increased in leaf along the altitude and showed a strong positive correlation with corresponding picrosides, suggesting higher accumulation of PI, PIII and PIV in leaf probably due to upregulation of MVA, MEP and phenylpropanoid pathways along the altitude. However, non detection of VA in leaf might indicates the absence of PII biosynthesis corroborating previous report (Kumar et al. 2017). In contrast to rhizome, a negative correlation among PII and VA in root indicates the possibility of an alternate route for PII biosynthesis that leads to PII accumulation in root along the altitude. Despite decreasing trends for PI, PIII and PIV in rhizome, increased accumulation of their corresponding phenolic acids (CA, FA and *p*-CA) were noticed along the altitude. Interestingly, no significant correlation was found among them suggesting flux of these aromatic acids to biosynthesis of other similar compounds such as 6-feruloyl-catalpol and specioside (Kumar et al. 2013). In addition, it also indicates that decreased content of picrosides (I, III and IV) in rhizome might be attributed to lesser transport from leaf and root to rhizome along the altitude. Furthermore, previous studies reported biosynthesis of PI and PII in organ specific manner; PI synthesizes in shoot, PII in root, whereas rhizome produce both (Kumar et al. 2017; Pandit et al. 2013), while no report has been available for PIII and PIV biosynthesis. Thus, this study suggesting PIII and PIV synthesized in both leaf and root and might be transported to rhizome for storage. However, further validation at the level of gene and protein will be required to test this hypothesis. Underground plant organs (rhizome and root) are utilized for medicinal purposes due to higher amount of PI and PII that



have been tested widely for their bioactivity (Sultan et al. 2017; Upadhyay et al. 2013; Dwivedi et al. 1992). Consistent with previous studies, this study also suggested that rhizome had maximum picroside content that could be used for future medical experiments. However, stress induced accumulation of picrosides at higher altitudes (3800 and 4100 masl) in leaf also suggests that they might be used as an alternative source for medical purposes. Iridoids accumulated in plants to provide defense against herbivores and pathogens. Previous study reported that high elevation and low temperature induced a lower level of iridoid as compared to lower elevation and high temperature in leaves of *P. lanceolata* due to decrease in herbivore pressure (Pellissier et al. 2014). Moreover, phenolics accumulation at high elevations reduces herbivore response, thereby, decreasing iridoid content (Pellissier et al. 2014). Contrastingly, Wang et al. (2010) suggested that drought stress resulted iridoid accumulation in roots of *S. ningpoensis*. Therefore, reduced biotic stress compensated by increased abiotic stress at higher altitude resulted in organ specific *P. kurroa* response and thus, contrasting trends of iridoid content were observed in leaf and underground organs i.e. root and rhizome.

## 5 Conclusions

The findings of our study shed light on metabolic response of herbal plants to environmental perturbations. It provides an insight into the biochemical processes associated with plant adaptation at high altitudes and better understanding of metabolic plasticity for alpine stress tolerance. We conclude that *P. kurroa* adaptation strategies along the altitude might involve modulation of sugar and amino acid metabolism, remodeling of root cell wall, ascorbate biosynthesis and cleavage, occurrence of partial TCA cycle, purine catabolism and salvage route, pyrimidine biosynthesis, alteration in lipid composition besides gibberellins and cytokinin inhibition. Reprogramming of primary metabolism directly influence SMs in organ specific manner and accumulation of protein, phenolics, flavonoids, cyanogenic glycosides, allelochemicals, glucosinolates, iridoid and picrosides may contribute effectively for *P. kurroa* adaptation at high altitudes. This study along with other omics tools might expand new avenues for metabolic engineering and stress tolerance in plants.

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## Compliance with ethical standards

**Conflict of interest** The authors have no conflicts of interest to declare.

**Ethical approval** This article does not contain any studies with human participants/animals performed by any of the authors.

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