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Contents of therapeutic metabolites in *Swertia chirayita* correlate with the expression profiles of multiple genes in corresponding biosynthesis pathways

Jibesh Kumar Padhan, Varun Kumar, Hemant Sood, Tiratha Raj Singh, Rajinder S. Chauhan *

Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat 173234, Solan, Himachal Pradesh, India

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ABSTRACT

Swertia chirayita, an endangered medicinal herb, contains three major secondary metabolites swertiamarin, amarogentin and mangiferin, exhibiting valuable therapeutic traits. No information exists as of today on the biosynthesis of these metabolites in *S. chirayita*. The current study reports the expression profiling of swertiamarin, amarogentin and mangiferin biosynthesis pathway genes and their correlation with the respective metabolites content in different tissues of *S. chirayita*. Root tissues of greenhouse grown plants contained the maximum amount of secoiridoids (swertiamarin, 2.8% of fr. wt and amarogentin, 0.1% of fr. wt), whereas maximum accumulation of mangiferin (1.0% of fr. wt) was observed in floral organs. Differential gene expression analysis and their subsequent principal component analysis unveiled ten genes (encoding HMGR, PMK, MVK, ISPD, ISPE, GES, G10H, 8HGO, IS and 7DLGT) of the secoiridoids biosynthesis pathway and five genes (encoding EPSPS, PAL, ADT, CM and CS) of mangiferin biosynthesis with elevated transcript amounts in relation to corresponding metabolite contents. Three genes of the secoiridoids biosynthesis pathway (encoding PMK, ISPD and IS) showed elevated levels (~57–104 fold increase in roots), and *EPSPS* of mangiferin biosynthesis showed an about 117 fold increase in transcripts in leaf tissues of the greenhouse grown plants. The study does provide leads on potential candidate genes correlating with the metabolites biosynthesis in *S. chirayita* as an initiative towards its genetic improvement.

1. Introduction

Swertia chirayita (Roxb. ex Fleming) H. Karst. (Family: Gentianaceae), commonly known as 'Chirata', is a native of temperate Himalayas, found at an altitude of 1200–3000 m (4000–10,000 ft.) from Kashmir to Bhutan and in the Khasia range (4000–5000 ft.) (Joshi and Dhawan, 2005). Chirayita is known by an array of names such as Anaryatikta, Bhunimba, Chiratitka, Kairata in Sanskrit, Chiaravata in Urdu, Qasabuzzarirah in Arab and Farsi and Chirrato or Chiraita in Nepalese. It has been reported in various pharmaceutical indexes (Joshi and Dhawan, 2005) and in Eastern traditional systems of medicine such as Ayurveda, Unani and Siddha (Williamson, 2002), which gives a clear impression of the widespread use and pharmacological importance of this biennial herb.

The broad-spectrum biological activities of Chirata are attributed to the presence of a diverse array of pharmacologically important secondary metabolites belonging to different classes such as xanthones and their derivatives, flavonoids, terpenoids, iridoids

Abbreviations: 7-DLGT, 7-deoxyloganetic acid glucosyl transferase; 8-HGO, 8-hydroxygeraniol oxidoreductase; AAC, acetyl-CoA carboxylase; AACT, acetoacetyl-CoA thiolase; ADH, arogenate dehydrogenase; ADT, prephenate dehydratase; C3H, *p*-coumarate 3-hydroxylase; C4H, trans-cinnamate 4-hydroxylase; CM, chorismate mutase; CS, chorismate synthase; DAHPS, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase; DHQD, 3-dehydroquinate dehydratase; DHQS, 3-dehydroquinate synthase; DL7H, 7-deoxyloganic acid hydroxylase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; G10H, geraniol 10-hydroxylase/8-oxidase; GDPS, geranyl diphosphate synthase; GES, geraniol synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; IO, iridoid oxidase; IPPI, isopentenyl diphosphate isomerase; IS, iridoid synthase; ISPD, 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase; ISPE, 4-(cytidine-5′-diphospho)-2-*C*-methyl-D-erythritol kinase; ISPF, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; ISPG, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; ISPH, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; LMT, loganic acid *O*-methyltransferase; MVDD, mevalonate diphosphate decarboxylase; MVK, mevalonate kinase; PAL, phenylalanine ammonia lyase; PAT, aspartate–prephenate aminotransferase; PHAT, phenylalanine (histidine) aminotransferase; PMK, phosphomevalonate kinase; SAK, shikimate kinase; SDH, shikimate dehydrogenase; SLS, secologanin synthase; TAL, tyrosine ammonia-lyase.

* Corresponding author. Tel.: +91 1792 239231.
E-mail address: rajinder.chauhan@juit.ac.in (R.S. Chauhan).

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and secoiridoid glycosides (Patil et al., 2013). The major phytochemicals of the bitter-tasting plant include swertiamarin, amarogentin (secoiridoid glucosides) and mangiferin, a xanthone C-glucoside (Phoboo et al., 2013). Swertiamarin is reported to be effective against hepatitis (Wang et al., 2011) and shown to exhibit anti-diabetic (Vaidya et al., 2013), anticancer (Kavimani and Manisenthlkumar, 2000) and anti-arthritic (Saravanan et al., 2014) activities. Amarogentin is known to be anti-diabetic (Phoboo et al., 2013), anticancer (Pal et al., 2012; Saha et al., 2006) and antileishmanial (Medda et al., 1999; Ray et al., 1996), whereas mangiferin has been tested for its anti-diabetic, anti-atherosclerotic (Pardo-Andreu et al., 2008), anticancer and anti-HIV (Guha et al., 1996) and antiparkinsonian (Kavitha et al., 2013) behaviors.

Overexploitation, due to an increased market demand along with other factors such as low seed viability (Badola and Pal, 2002) and narrow geographic occurrence (Bhat et al., 2013) has led to the endangered status of plant. The molecular understanding of biosynthesis routes leading to the production of major chemical constituents is lacking.

Swertiamarin and amarogentin take the classical MVA/MEP route of terpene biosynthesis followed by the secoiridoid pathway (Fig. 1). These secoiridoids originate from a common intermediate sweroside, which is biosynthesized from geranyl diphosphate (GPP), a condensation product of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Then they are transformed to the end products through a cascade of chemical conversions along with the formation of intermediates such as irido-dial/trial, deoxyloganic acid, loganic acid and secologanic acid (Miettinen et al., 2014; Wang et al., 2001). Swertiamarin is the hydroxylation product of sweroside, whereas amarogentin is the biphenylcarboxylic acid derivative of the same. The biphenylcarboxylic acid moiety was presumed to be biosynthesized from *m*-hydroxybenzoyl-CoA by a polyketide-type pathway from phenylalanine via cinnamic acid and benzoic acid as reported in *Swertia japonica* (Kuwajima et al., 1990). A retrobiosynthetic ^{13}C NMR study on *S. chirayita* also proved the formation of biphenylcarboxylic acid moiety from *m*-hydroxybenzoic acid, the later being formed preferentially from an early shikimate pathway intermediate, rather than from phenylalanine via cinnamic acid and benzoic acid (Wang et al., 2001). However, minor fractions of phenylalanine-derived metabolites might also contribute to amarogentin biosynthesis, which awaits an experimental proof. Mangiferin, on the other hand, follows a combined phenylpropanoid/acetate route (Fig. 2), where it is biosynthesized from phenylalanine through *p*-coumaric acid/cinamic acid and malonic acid along with benzophenone intermediates such as iriflophenone and maclurin as described in *Anemarrhena asphodeloides* (Fujita and Inoue, 1977).

Apart from a few studies as to expression analysis and functional characterization of geraniol 10-hydroxylase (G10H), a P450 in the CYP76B subfamily in *Swertia mussotii*, no comprehensive study has been done to associate the expression status of swertiamarin, amarogentin and mangiferin pathway genes with their biosynthesis in *S. chirayita*. We report for the first time the expression analysis of 24 genes of swertiamarin/amarogentin

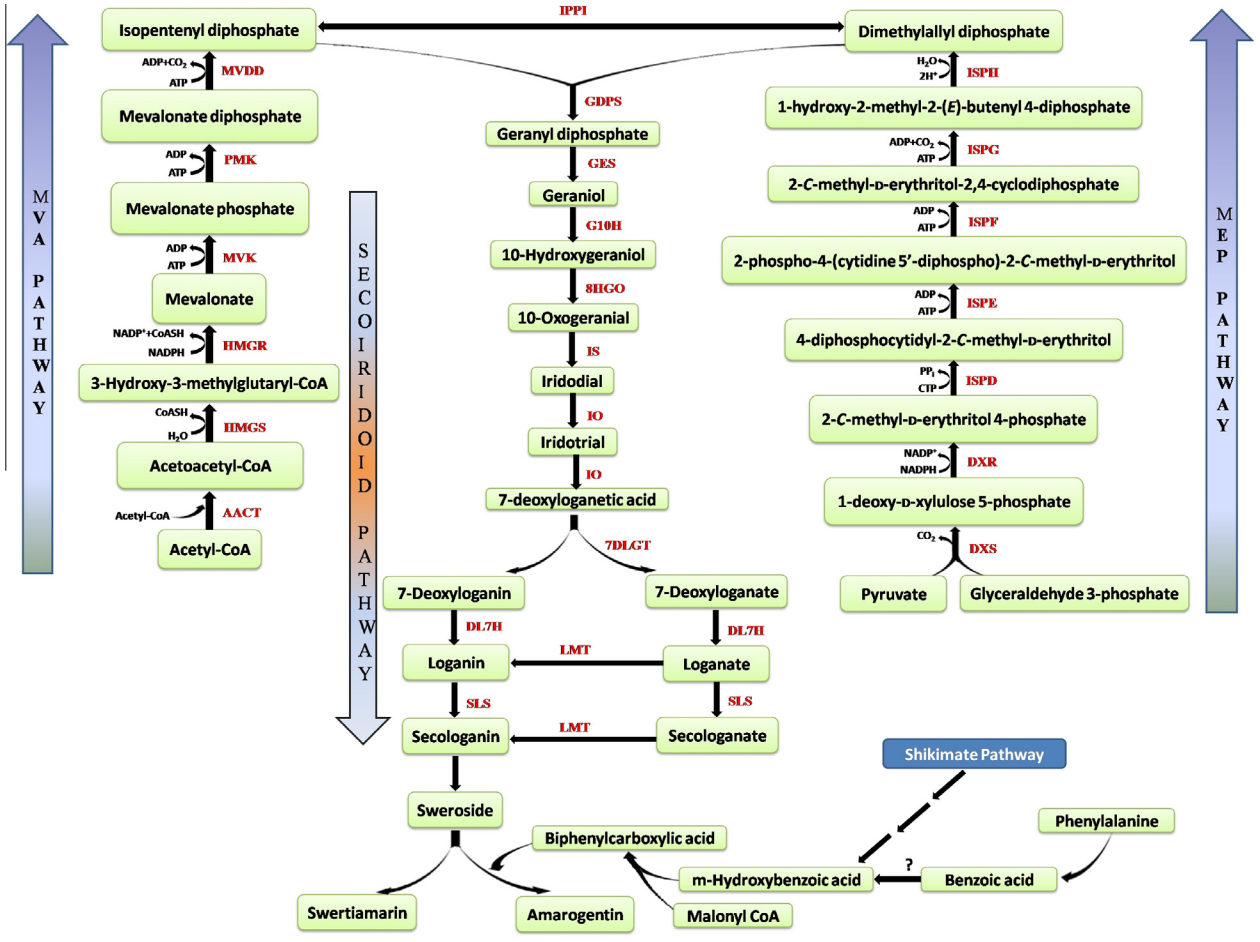

Fig. 1. Schematic representation of the proposed swertiamarin–amarogentin biosynthesis pathway (partly adapted from Miettinen et al. (2014)).

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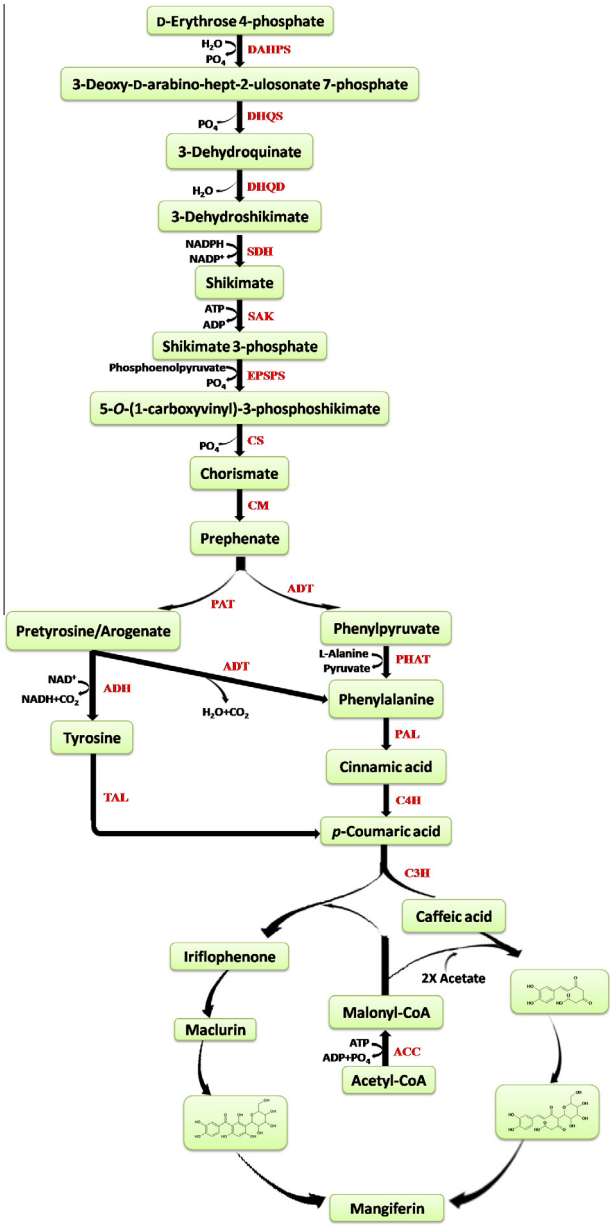

Fig. 2. Schematic representation of the proposed mangiferin biosynthesis pathway.

biosynthesis and 15 genes of the mangiferin biosynthesis route in 5 different tissues of *S. chirayita* varying in contents of major chemical constituents.

2. Results and discussion

2.1. Differential biosynthesis and accumulation of swertiamarin, mangiferin and amarogentin

Swertiamarin, mangiferin and amarogentin content across all tissues ranged from 0.1% to 2.8% of fresh weight (fr. wt), 0.02% to 1.0% of fr. wt and 0.01% to 0.1% of fr. wt, respectively (Figs. 3 and 4). Among all tissues, roots of greenhouse grown plant (GHR) showed the highest content of swertiamarin (2.8% of fr. wt) and amarogentin (0.1% of fr. wt). These amounts were 57 and 10 folds higher in comparison to roots of tissue cultured *Swertia* (TCR) that contained 0.1% and 0.01% of swertiamarin and amarogentin, respectively (Table 1). The flowers from greenhouse grown plants (GHF) contained a higher amount of swertiamarin (1.7% of fr. wt) and amarogentin (0.04% of fr. wt) compared to leaf tissues of the same plants (GHL), which were estimated to be 0.6% and 0.03% of fr. wt, respectively. The leaves of tissue cultured plant (TCL) contained a higher amount of swertiamarin (0.3% of fr. wt) and amarogentin (0.1% of fr. wt) in comparison to its tissue cultured counterpart. Similarly, maximum mangiferin content (1.0% of fr. wt) was in GHF, which was 49-fold higher compared to GHR (0.02% of fr. wt) and roots of tissue cultured plants, TCR (0.02% of fr. wt). Also, mangiferin content in GHL was 0.7% of fr. wt, whereas in TCL, the xanthone content was estimated to be 0.4% of fr. wt. From this study it is inferred that the biosynthesis and accumulation of secondary metabolites is tissue and organ specific and dependent on environmental conditions. This observation is well supported by previous studies which showed differential accumulation of picroside-I and picroside-II in different tissues of *Picrorhiza kurroa* (Pandit et al., 2013) and accumulation of artemisinin in *Artemisia annua* L. (Olofsson et al., 2011).

2.2. Relative expression of pathway genes in relation to metabolite contents

In order to better understand the molecular basis of biosynthesis of swertiamarin, amarogentin and mangiferin, quantitative expression analysis of 24 genes of swertiamarin/amarogentin and 15 genes of mangiferin biosynthesis were carried out in 5 different tissues of *S. chirayita* through qRT-PCR. The first notable outcome of the gene expression analysis gave an indication that the secoiridoid pathway genes were mostly active and strongly expressed in the tissues collected from the greenhouse plants in comparison to tissue cultured plants. However, this pattern was not observed in the mangiferin pathway genes. In roots of greenhouse grown *Swertia* (GHR), which showed the highest amount of swertiamarin and amarogentin, 16 genes of the secoiridoids biosynthesis pathway, encoding DL7H, IS, ISPD, PMK, LMT, MVK, GES, 8HGO, G10H, GDPS, ISPH, 7DLGT, IO, ISPE, IPPI and HMGR showed elevated levels of expression ($\geqslant$3-fold) in comparison to TCR, with the least content of secoiridoids (Fig. 5 and Table 2). Among these genes, *DL7H* showed 273 fold, *IS* showed 105 fold, *ISPD* showed 102 fold, *PMK* 57-fold, *LMT* 30-fold, *MVK* 27-fold, *GES* 24-fold, *8HGO* 20-fold, *G10H* 19-fold, *GDPS* 15-fold, *ISPH* 12-fold, *7DLGT* 8-fold, *ISPE* 6-fold and *HMGR* 3-fold increase in transcript abundance. A similar pattern was inferred from leaves of greenhouse grown *Swertia* (GHL), having 11 and 3-fold higher swertiamarin and amarogentin content, respectively, than TCR, which showed 13 genes (encoding MVK, PMK, DXR, ISPD, ISPE, ISPF, IS, LMT, ISPH, IPPI, GDPS, G10H and 8HGO) with more than 3-fold increase in gene expression. Among these genes encoding 8HGO, IS, ISPH, PMK, GDPS, LMT, G10H, IPPI, ISPF, DXR, MVK, ISPD and ISPE showed 35, 22, 16, 15, 13, 10, 9, 8, 7, 7, 5, 4 and 3-fold change in gene expression, respectively. Also, 4 genes (encoding ISPH, 8HGO, DL7H and SLS) showed an increase in gene expression level ($\geqslant$3-fold) in TCL with respect to TCR. In contrast to these, the floral tissue GHF, showed only 2 genes (coding for ISPH and 8HGO) with more than 3-fold gene expression despite a higher level of swertiamarin and amarogentin (33 and 4-fold, respectively w.r.t TCR). In order to filter out the important genes of the biosynthesis pathway, a comparative study of expression of different genes of the metabolic pathways across different tissues was performed (Fig. 6). The analysis showed that the expression of 4 genes (encoding MVK, PMK, GES and LMT) in the swertiamarin/amarogentin biosynthesis varied in accordance with the metabolite concentrations in different tissues.

The expression analysis of 15 genes participating in the mangiferin biosynthesis revealed some correlation of a number of genes in tune with the metabolite contents and the tissue types (Fig. 7 and Table 3). In GHR, 10 genes of the metabolic pathway (coding for DAHPS, DHQS, DHQD, EPSPS, CS, CM, ADT, PAL, C4H and AAC)

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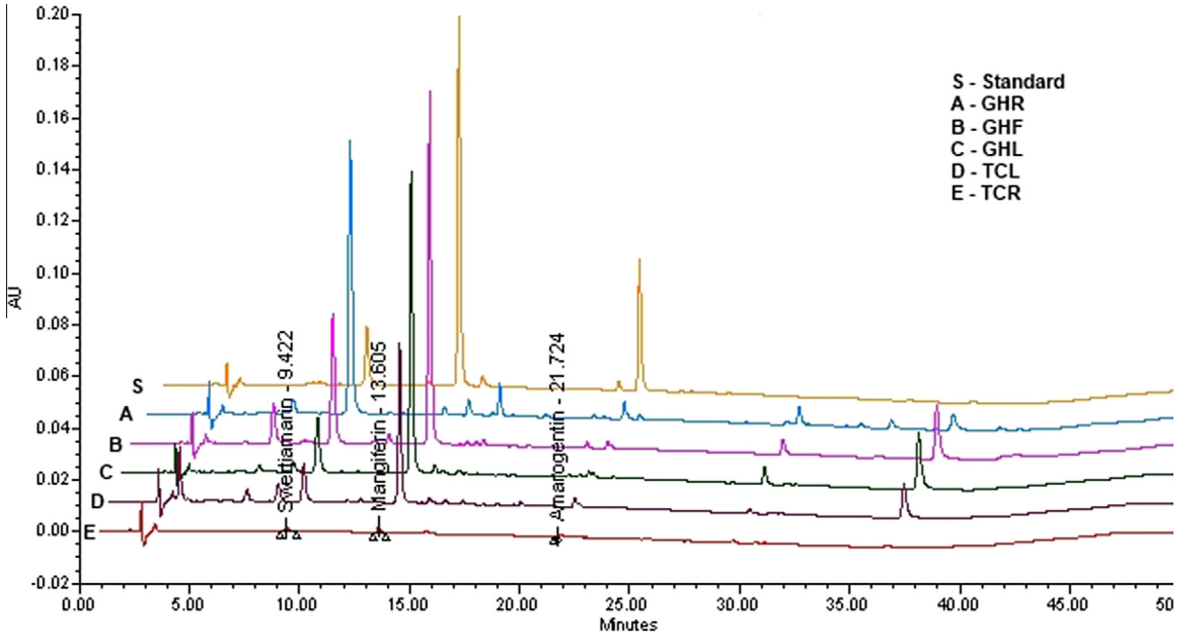

Fig. 3. HPLC profile of swertiamarin, mangiferin and amarogentin in different tissues of *Swertia chirayita*. GHR, roots of greenhouse grown *Swertia*; GHF, flowers of greenhouse grown *Swertia*; GHL, leaves of greenhouse grown *Swertia*; TCL, leaves of tissue cultured *Swertia*; TCR, roots of tissue cultured *Swertia*; S, standard.

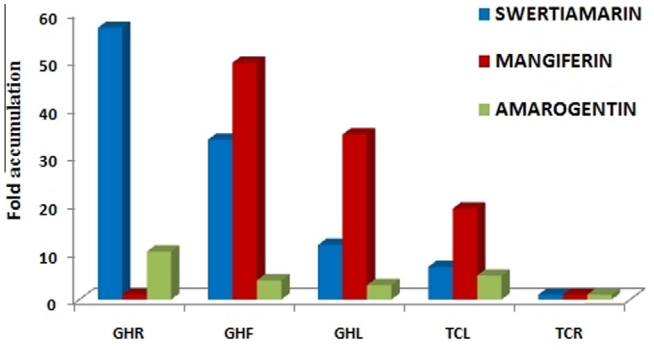

Fig. 4. Fold difference in swertiamarin, mangiferin and amarogentin contents in different tissues w.r.t. TCR. GHR, roots of greenhouse grown *Swertia*; GHF, flowers of greenhouse grown *Swertia*; GHL, leaves of greenhouse grown *Swertia*; TCL, leaves of tissue cultured *Swertia*; TCR, roots of tissue cultured *Swertia*.

showed increased levels ($\geqslant$3-fold) of expression in defiance of the low content of mangiferin. The transcript abundance of 8 genes (encoding EPSPS, DHQS, PAL, ADT, CM, DAHPS, DHQD and CS) increased in relation to mangiferin content in leaves. However, in floral tissue transcript abundance of most of the genes except *EPSPS*, dropped in spite of mangiferin content being the highest, thereby suggesting that the floral tissue may be a site of mangiferin accumulation rather biosynthesis.

2.3. Principal component analysis

Principal component analysis (PCA) revealed the relationship between each module and their projections have been observed for 5 different conditions for 4 component combinations. It has been observed that major variance was generated by principle components F1 and F2 (Fig. 8A and B). PCA analysis unveiled 10 genes of secoiridoid biosynthesis (encoding HMGR, GES, 7DLGT, 8HGO, ISPD, ISPE, PMK, MVK, IS and G10H) and 5 genes (coding for EPSPS, CS, PAL, CM and ADT) of mangiferin biosynthesis as the major contributors. In addition to that, genes encoding SLS, DL7H, AACT and DXS (secoiridoids biosynthesis pathway) and C4H, ADH, AAC, PAT, SDH and PHAT (mangiferin biosynthesis pathway), also contributed towards their respective metabolites biosynthesis based on their squared cosine values in PCA, which is in coordination with the relative expression analysis of genes and their relation to metabolite contents.

The overall study identified certain prime gene candidates encoding HMGR, PMK, MVK, ISPD, ISPE, GES, G10H, 8HGO, IS and 7DLGT of the secoiridoids biosynthesis pathway and 5 genes (coding for EPSPS, PAL, ADT, CM and CS) of mangiferin biosynthesis which significantly contribute to the biosynthesis of three metabolites. Our study was analogous to previous reports, where secondary metabolites like picrosides in *P. kurroa* (Pandit et al., 2013), shikonin in *Arnebia euchroma* (Singh et al., 2010) and aconites in *Aconitum heterophyllum* (Malhotra et al., 2014) have been shown to be positively correlated with the expression profile of multiple genes of their respective metabolic pathways. Higher expression levels of *ISPD* and *ISPE* have been associated with higher picroside-I content in *P. kurroa* (Pandit et al., 2013). Similar expression studies revealed the role of *HMGR* in *A. annua* (Alam and Abdin, 2011), where overexpression of the gene enhanced the production of artemisinin. The importance of *PMK* and *MVK* genes has been well illustrated in previous studies, where they were targeted to enhance the overall isoprenoid production (Singh et al., 2012). Previous study indicated the importance of *G10H*, as overexpression of *G10H* in the hairy roots has improved production of several MIAs (Peebles et al., 2011). The significance of gene encoding GES in geraniol production has been described in a previous report, where they optimized geraniol production based on expression of plastid-targeted *GES* from *Valeriana officinalis* in transgenic tobacco suspension cultures (Vasilev et al., 2014).

Apart from the leaves, expression profiling of genes involved in mangiferin biosynthesis did not show any specific pattern in relation to mangiferin content across the investigated tissues. This is in accordance with a previous report, where the expression levels of the genes involved in the general phenylpropanoid pathway did not show significant correlation with the accumulation pattern of shikonin (Yamamura et al., 2003). This may suggest that the highly expressed genes are involved in the biosynthesis of other

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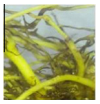
Table 1
Variation in contents of swertiamarin, amarogentin and mangiferin across different tissues of *S. chirayita*. GHR, roots of greenhouse grown *Swertia*; GHF, flowers of greenhouse grown *Swertia*; GHL, leaves of greenhouse grown *Swertia*; TCL, leaves of tissue cultured *Swertia*; TCR, roots of tissue cultured *Swertia*. % – Percentage of fresh weight (fr. wt) and values are mean ± SEM.

Tissue	Source	Swertiamarin (%)	Amarogentin (%)	Mangiferin (%)	
GHR	Greenhouse	2.8 ± 0.13	0.1 ± 0.01	0.02 ± 0.00	
GHF	Greenhouse	1.7 ± 0.09	0.04 ± 0.00	1.0 ± 0.07	
GHL	Greenhouse	0.6 ± 0.06	0.03 ± 0.00	0.7 ± 0.05	
TCL	Tissue cultured	0.3 ± 0.07	0.1 ± 0.00	0.4 ± 0.01	
TCR	Tissue cultured	0.1 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	

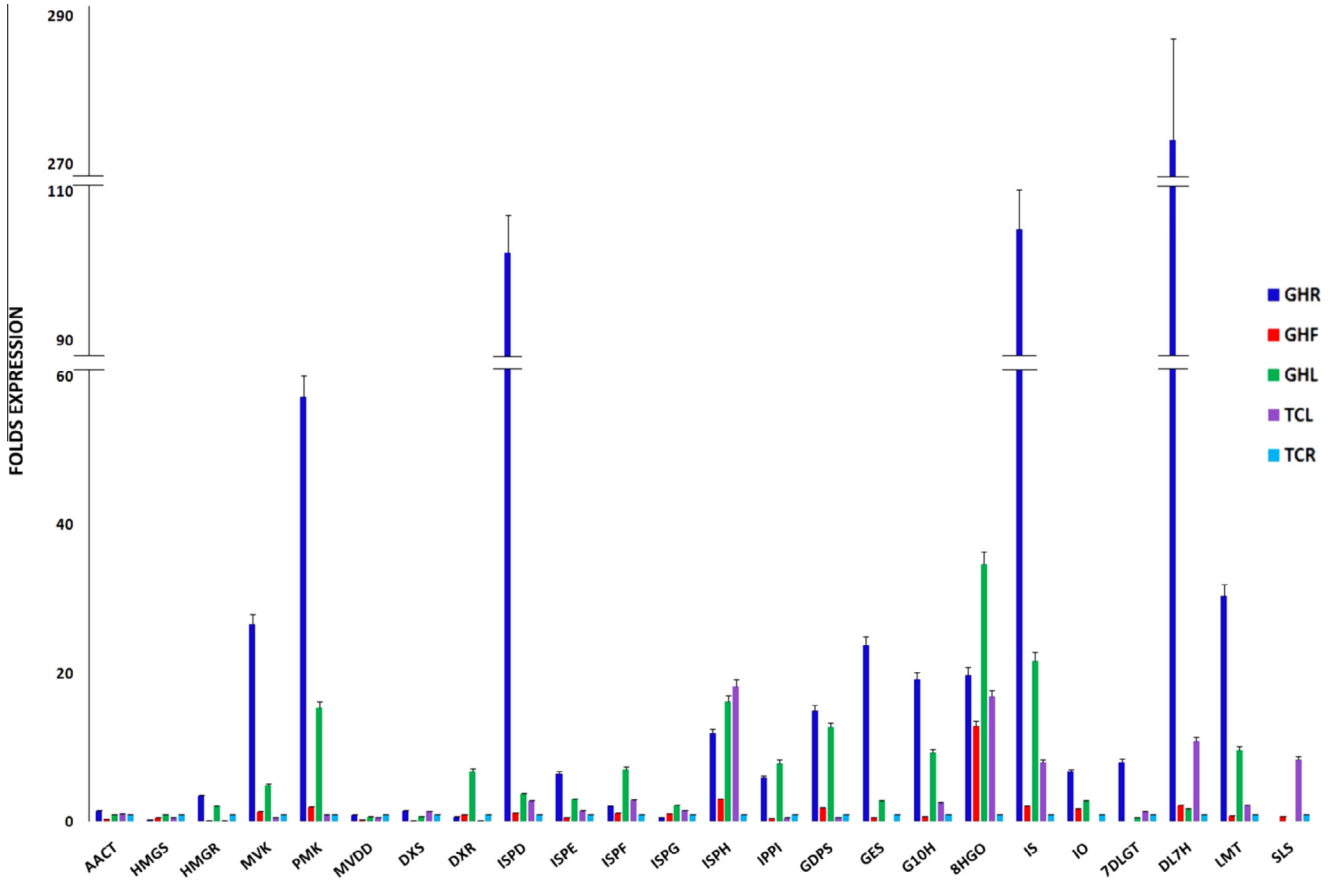


Fig. 5. Relative fold changes in the expression of swertiamarin/amarogentin biosynthesis pathway genes across different tissues w.r.t. TCR. GHR, roots of greenhouse grown *Swertia*; GHF, flowers of greenhouse grown *Swertia*; GHL, leaves of greenhouse grown *Swertia*; TCL, leaves of tissue cultured *Swertia*; TCR, roots of tissue cultured *Swertia*.

secondary metabolites. In consistent with our finding, a previous study also vouched the importance of the versatile *PAL* gene, whose expression varied in accordance with condensed tannins (CT) and lignin biosynthesis in developing shoot and root tissues of *Populus tremuloides* Michx. (Kao et al., 2002). The *EPSPS* gene which emerged as a potential regulatory gene in our study has been reported to be a significant player in the aromatic amino acid biosynthesis pathway being used as glyphosate target (Tian et al., 2013). The floral tissue did not show correlation of the expression pattern of pathway genes in relation to their metabolite contents.

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Table 2
Relative fold expression of secoiridoid biosynthesis pathway genes across different tissues with respect to roots of tissue cultured *Swertia* (TCR).

		Greenhouse roots (GHR)	Greenhouse flowers (GHF)	Greenhouse leaves (GHL)	Tissue cultured leaves (TCL)
1	AACT	–	–	–	–
2	HMGS	–	–	–	–
3	HMGR	+	–	–	–
4	MVK	++	–	+	–
5	PMK	+++	–	++	–
6	MVDD	–	–	–	–
7	DXS	–	–	–	–
8	DXR	–	–	+	–
9	ISPD	++++	–	+	–
10	ISPE	+	–	+	–
11	ISPF	–	–	+	–
12	ISPG	–	–	–	–
13	ISPH	++	+	++	++
14	IPPI	+	–	+	–
15	GDPS	++	–	++	–
16	GES	++	–	–	–
17	G10H	++	–	+	–
18	8HGO	++	++	++	++
19	IS	++++	–	++	+
20	IO	+	–	–	–
21	7DLGT	+	–	–	–
22	DL7H	++++	–	–	++
23	LMT	++	–	+	–
24	SLS	–	–	–	+

Scale: <3-fold (–), ⩾3–10-fold (+), ⩾10–40-fold (++), ⩾40–80 (+++), ⩾80 (++++).

This may suggest the involvement of a transport mechanism wherein the metabolites are synthesized outside the sink-tissue and are later transported to floral tissue, in a similar manner like nicotine gets transported from roots to the central vacuoles of leaves by Nt-JAT1, a (MATE)-type transporter in *Nicotiana tabacum* (Shitan et al., 2009), and transport of glucosinolates from maternal tissue to seeds involving NRT/PTR transporters of *Arabidopsis thaliana* (Nour-Eldin et al., 2012).

All these reports strongly support the experimental outcomes of our study and advocate the prime candidacy of these genes for being considered as suitable targets for improving metabolic traits. These key genes with high transcript abundance in relation to their metabolite contents can be targeted for overproduction of the desired phytochemicals. For instance, overexpression of genes encoding HMGR, DXS and prenyltransferases has been shown to elevate terpenoid levels in various plant species (Degenhardt et al., 2003). An alternative to engineer individual pathway genes is to identify and modulate expression of transcription factors. These transcription regulators steer the metabolic flux of secondary metabolite biosynthesis often controlling the expression of blocks of genes of the pathway. In undifferentiated maize cells cultured *in vitro*, induction of complete flavonoid pathway has been reported by the overexpression of a combination of two transcription factors, R and C1 (Grotewold et al., 1998). On the contrary, overexpression of *FaMYB*, a MYB-type transcription factor from strawberry has led to a reduction in flower pigmentation along with decreased levels of anthocyanin and flavonol compounds (Aharoni et al., 2001). This suggests a careful study of the detailed regulatory control circuit in metabolic engineering process while targeting transcription factors as regulators.

3. Concluding remarks

The quantitative gene expression in relation to swertiamarin, amarogentin and mangiferin content across different tissues of *S. chirayita* grown under differential environmental conditions unveiled key genes of secoiridoids (encoding HMGR, PMK, MVK, ISPD, ISPE, GES, G10H, 8HGO, IS and 7DLGT) and mangiferin (coding for EPSPS, PAL, ADT, CM and CS) biosynthesis pathway. However, the outcome requires further corroboration through biochemical and enzymological studies like incorporation/inhibition experiments to infer how the final enzyme levels change with an increase in the corresponding transcripts amounts. Also, a thorough study on different kinetic parameters determining the enzymatic turn over, synthesis and degradation of end products and

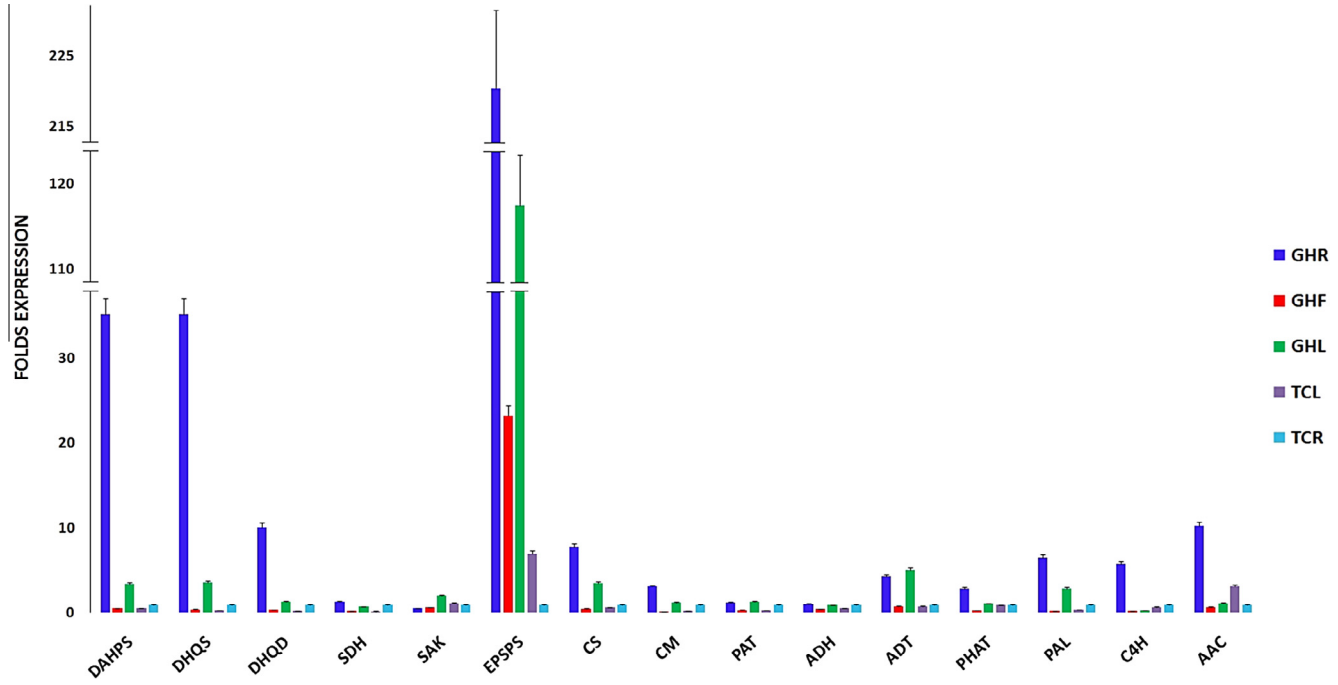

Fig. 6. A comparative account of expression of pathway genes across different tissues. GHR, roots of greenhouse grown *Swertia*; GHF, flowers of greenhouse grown *Swertia*; GHL, leaves of greenhouse grown *Swertia*; TCL, leaves of tissue cultured *Swertia*; TCR, roots of tissue cultured *Swertia*.

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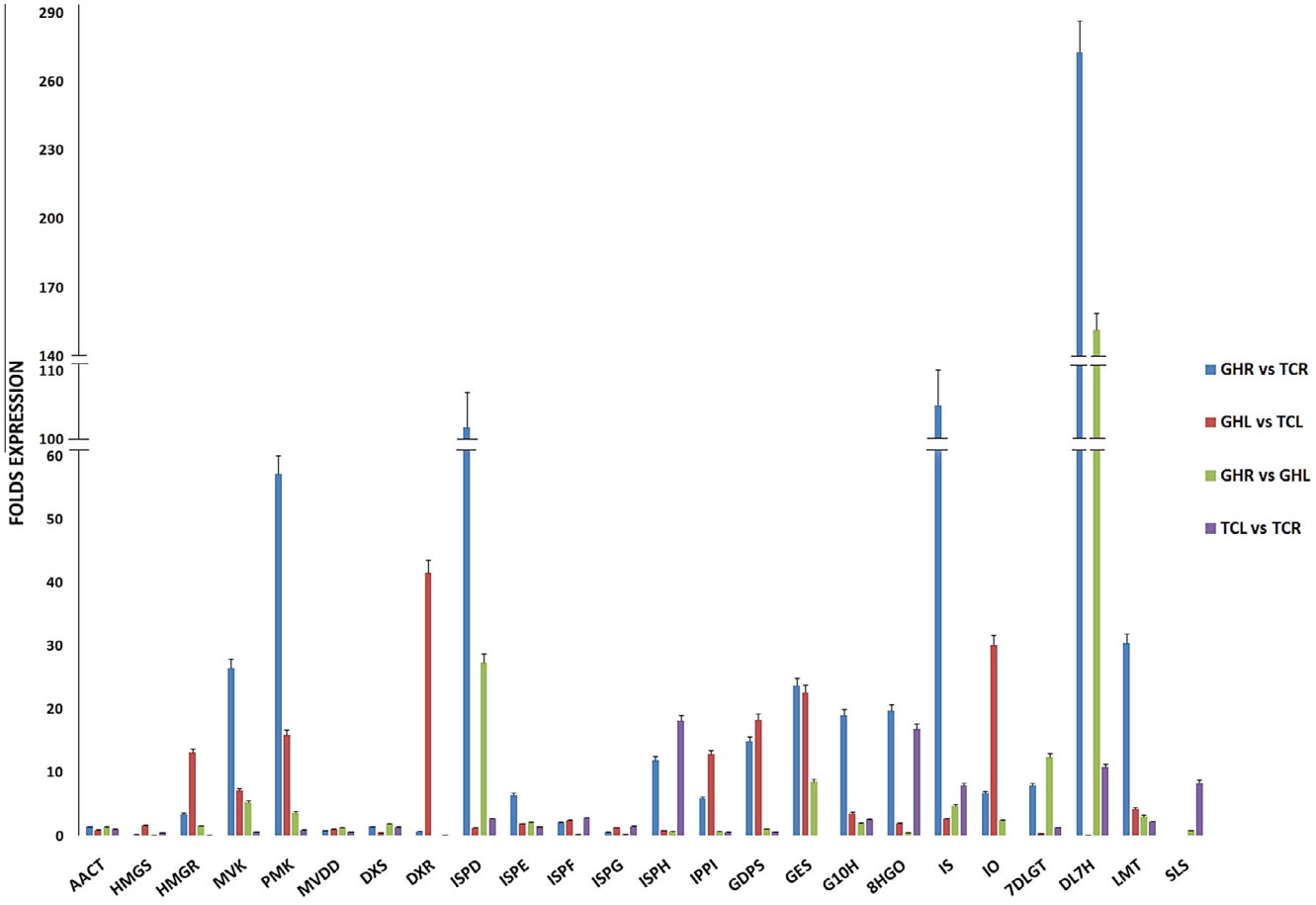

Fig. 7. Relative fold changes in the expression of mangiferin biosynthesis pathway genes across different tissues w.r.t. TCR.

Table 3
Relative fold expression of mangiferin biosynthesis pathway genes across different tissues with respect to roots of tissue cultured *Swertia* (TCR).

		Greenhouse roots (GHR)	Greenhouse flowers (GHF)	Greenhouse leaves (GHL)	Tissue cultured leaves (TCL)
1	DAHPS	++	–	+	–
2	DHQS	++	–	+	–
3	DHQD	++	–	–	–
4	SDH	–	–	–	–
5	SAK	–	–	–	–
6	EPSPS	++++	++	++++	+
7	CS	+	–	+	–
8	CM	+	–	–	–
9	PAT	–	–	–	–
10	ADH	–	–	–	–
11	ADT	+	–	+	–
12	PHAT	–	–	–	–
13	PAL	+	–	–	–
14	C4H	+	–	–	–
15	AAC	++	–	–	+

Scale: <3-fold (–), ⩾3–10-fold (+), ⩾10–40-fold (++), ⩾40–80 (+++), ⩾80 (++++).

hence, the overall metabolic flux need to be examined to gain a better understanding of the underlying regulatory mechanisms involved. Regulatory surveillance by various transcription factors and epigenetic determinants controlling the levels of major phytochemicals in *S. chirayita* need to be investigated as illustrated in carotenogenesis (Cazzonelli and Pogson, 2010). Characterization and functional analysis of promoter regions can provide a clear picture of the differential expression status of these genes in different tissues. Identification of key genes with high transcript abundance in relation to metabolite content is an initiative towards the development of gene markers for genetic improvement of *S. chirayita* with enhanced secondary metabolite production.

4. Experimental

4.1. Plant material and tissue collection

Plants of *S. chirayita* were maintained in a greenhouse at the Jaypee University of Information technology, Waknaghat, H.P., India (1500 m altitude; 31°01′N, 77°04′E) with controlled environmental conditions [light (intensity 300–2000 lx), temperature (25 ± 2 °C), relative humidity (≈75%) and photoperiod (14 h day/10 h night)]. Tissue cultured *S. chirayita* plants were maintained at 25 ± 2 °C in media supplemented with MS (Murashige and Skoog, 1962) salts, growth hormones [Kinetin (2 mg/l), Indole-3-butyric acid (2 mg/l) and gibberellic acid (3 mg/l)], sucrose (30 g/l), agar–agar (8.5 g/l) pH 5.6–5.7 and controlled environmental conditions [light (intensity 2000 lx), temperature (25 ± 2 °C), relative humidity (≈75%) and photoperiod (16 h day/8 h night)]. Tissue samples from 2-year-old greenhouse plants (roots, leaves and flowers) and 1.5 month old tissue cultured plants (roots and leaves) were collected, immediately frozen in liquid nitrogen and stored at −80 °C until further use.

4.2. Extraction and estimation of swertiamarin, mangiferin and amarogentin

Swertiamarin, mangiferin and amarogentin contents were estimated simultaneously by reverse phase High Performance Liquid

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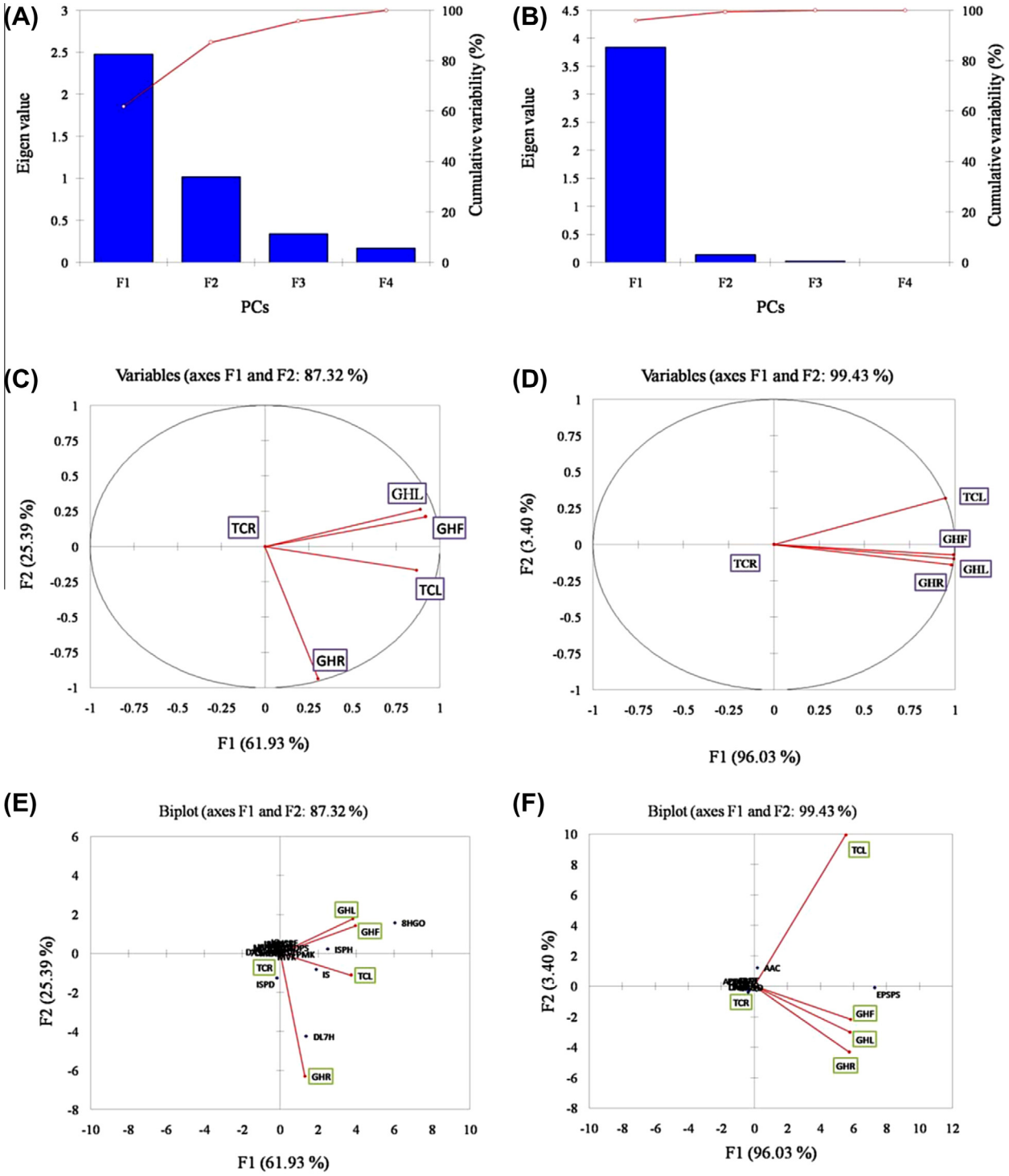

Fig. 8. PCA: A and B screen plot for principal components (F1–F4), their respective Eigen values, and cumulative variability. These four components are the result of five tissue types through dimensionality reduction. Major variance was contributed by components 1 and 2 in A, whereas in B, major variance was contributed by component 1. PCA is successful when major contribution is from first two components which is clearly visible from the screen plots. (C) and (D) Variable plot of PCA for the secoiridoids (C) and mangiferin (D) biosynthesis pathway. As major contribution came from first two components, variable plots for F1 and F2 were generated. Overall contribution of F1 and F2 in C is 87.32%, while in D it is 99.43%. This difference in variability came through the position of GHR tissue. (E) and (F) Biplot for the PCA analysis of five different tissue types (TCR, TCL, GHR, GHF and GHL). (E) Shows observations for 24 genes of the secoiridoids biosynthesis pathway, whereas F represents biplot for 15 genes of mangiferin biosynthesis. The overall PCA revealed 10 and 5 key genes from the secoiridoids and the mangiferin biosynthesis pathways, respectively.

Chromatography (HPLC Waters 515) as described previously (Kumar et al., 2013). The plant materials were ground in liquid nitrogen and suspended in 80% methanol. The sample mixtures were sonicated for 10 min. The following day, the samples were centrifuged at 10,000 rpm for 15 min. The supernatant was then filtered through 0.22 μm filter. The desired separation and baseline was obtained in a gradient method, where mobile phase A was composed of 0.1% TFA in water and mobile phase B was a mixture of acetonitrile/water in a ratio of 70:30. The linear gradient at a flow rate of 1.0 ml/min. was started with 15% B; 20% B in next

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5 min., 70% B in next 25 min., hold for 5 min.; 15% B in next 5 min., equilibrated for 5 min. and detected at 240 nm UV wavelength. The separation was obtained on Waters Spherisorb 5 μm ODS2 (250 mm × 4.6 mm). The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards procured from ChromaDex (Irvine, CA). The quantification was done in triplicate and the data were interpreted in terms of % of fresh weight (fr. wt) of swertiamarin, mangiferin and amarogentin.

4.3. Isolation of total RNA

Total RNA was extracted from the aforementioned tissues using Trizol Reagent (Ambion), according to manufacturer's instructions. Quality, purity and concentration of RNA were estimated spectrophotometrically by A260 and A280 measurements (NanoDrop, Thermo Scientific) followed by visualizing on ethidium bromide-stained agarose gel.

4.4. Expression analysis of MVA/MEP, secoiridoid and phenylpropanoid/acetate pathway genes through quantitative PCR (qPCR)

Total RNA (1 μg) was used to synthesize first strand cDNA using Verso cDNA synthesis kit with anchored oligo(dT) primers and Verso RT Enhancer (Thermo Scientific, Wilmington, DE) to remove residual DNA contamination, following the manufacturer's protocol. Gene sequences of the MVA/MEP, secoiridoid and phenylpropanoid/acetate pathways were retrieved from the whole transcriptome data of *S. chirayita* (Unpublished) using BLAST similarity search program (http://www.ncbi.nlm.nih.gov/BLAST). Gene specific primers were designed from the retrieved gene sequences using Primer3 (Rozen and Skaletsky, 2000). Multiple internal control genes were used to normalize the expression data as previously described (Vandesompele et al., 2002). A set of six candidate reference genes encoding actin, eEF1a, β-tubulin, histone H3, 26s rRNA and GAPDH (Supplementary Table 1) were taken into consideration for their expression across different tissues under study and their suitability as reference genes were analyzed using BestKeeper (Pfaffl et al., 2004). qPCR reactions were run on a CFX96 system (Bio-Rad Laboratories, Hercules CA) with the iScript one step RT PCR kit (Bio-Rad). The cycling conditions comprised denaturation for 5 min. at 94 °C, followed by 40 cycles each of denaturation for 20 s at 94 °C, annealing for 30 s at different temperatures (Supplementary Table 1), followed by elongation step for 20 s at 72 °C. All reactions were run in triplicate. Relative fold changes were determined from the Cq values using the comparative Ct (ΔΔCt) method as previously described (Schmittgen and Livak, 2008). The specificity of assay was evaluated by melting curve analysis comprising melting curve ramping from 65 °C to 95 °C with an increasing temperature of 0.5 °C for 5 s (1 cycle).

4.5. Statistical analysis

Principal component analysis (PCA) (Hotelling, 1933) was performed to determine the relationship between each modules of TCR, TCL, GHR, GHF, and GHL and their assessments for all the five conditions for all 24 genes of the swertiamarin and amarogentin biosynthesis pathway and 15 genes of mangiferin biosynthesis. Plot between variability, Eigen-values and principal components were generated (Fig. 8A and B). Again, variable plots (Fig. 8C and D) and biplots (Fig. 8E and F) for the same were generated so as to represent the exact distribution of all the genes involved and their contribution towards experimental conditions.

Author contributions

RSC and JKP contributed towards conception and design of research theme, analysis and interpretation of result, manuscript writing and critical revision of the article. JKP carried out the laboratory experiments and analyzed the data. VK carried out HPLC analysis. HS helped in co-designing of the experiment and critical revision of the article. TRS performed the principal component analysis and interpretation of data. All authors have contributed to, seen and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.05.007.

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