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Effect of contrasting climates on antioxidant and bioactive constituents in five medicinal herbs in Western Himalayas

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Abstract: To understand the effect of climate change on constitutive antioxidant and biochemical metabolites in Western Himalayas, five medicinal herbs were selected and grown at two altitudes in Jammu (305 m) and Srinagar (1730 m) with subtropical and temperate climates, respectively. Significant variations were observed in phenols and flavonoids in *Hypericum perforatum* L., *Matricaria chamomilla* L., *Thymus vulgaris* L., *Cynara cardunculus* L. and *Echinacea purpurea* L. growing at two locations. High altitude temperate site show variable (up to 13 fold) increase in their content. Proteins (1.3 - 1.8 times), sugars (2.8 - 4.1 times) and free amino acid (1.04 - 1.22 times) were also higher at Srinagar (1730 m). Within these plants, *H. perforatum* and *M. chamomilla* have shown higher accumulation of phenols, xanthophylls and proline even at subtropical environment in Jammu (305 m) suggesting potential for increasing their geographical area. The results demonstrate that changing environmental conditions significantly affect the bio-active constituents, which accumulate as a defence strategy by these temperate plants. Their medicinal significance during climate change scenario has also been discussed.

Keywords: Medicinal herbs; Flavonoids; Phenols; Glutathione; Western Himalayas; Climate change

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Introduction

Plants contain an enormous variety of chemical compounds known as secondary metabolites that are used for specific odours, tastes and colours. Most of the medicinal properties of plants have been attributed to these compounds and have been utilized by the human race from time immemorial. However, these compounds help plants respond to environmental stimuli in a rapid, reversible and ecologically meaningful manner and thus, plays crucial role in existence of plants in any environment (Metlen et al. 2009). In other words, the environmental factors play an important role in regulating the metabolic content of these bioactive molecules, and dynamic response of these compounds is one of the factors defining plant's adaptation strategy (Silvertown 1998; Tuteja and Sopory 2008). Being sessile, one of the most challenging tasks for plants is to combat the unfavorable conditions that can alter or reduce qualitative and quantitative yield. These molecules such as phenols, flavonoids, anthrocyanin, carotenoids, non-protein amino acids together with low molecular weight molecules such as glutathione and ascorbate help the plant to combat stress by acting as scavengers for reactive oxygen species (ROS) or by regulating its cellular redox

environment (Potters et al. 2010). Most of the medicinal and antioxidant properties in medicinal plants have been associated with these bioactive molecules. It is widely believed that plants growing under stressful environments produce higher and better quality of these bioactive compounds (Ramakrishna and Ravishankar 2011; Hartmann 2007). Generally when plants are stressed, secondary metabolite production may increase because growth is often inhibited more than photosynthesis, and the fixed carbon is not allocated to growth and instead allocated to secondary metabolites (Mooney et al. 1991).

The Indian Himalayan region has a rich flora of medicinal and aromatic plants and so far 1748 species have been reported that are medicinally important (Samant et al. 1998). These plants are source of traditional plant based medicines on which about 70% of population of India is dependent (Gadgil and Rao 1998). Its enriched biodiversity is expanded from Afghanistan to China and is characterized by a marked altitudinal, geographical and climatic variation representing different microclimatic zones viz. subtropical, tropical, temperate, alpine and cold desert. Recent patterns of climate change have indicated that the Himalaya and surrounding areas have been the most affected and requires urgent attention (Gairola et al. 2010). In Himalayan region, particularly in alpine areas, changes in precipitation patterns and temperatures are already affecting the distribution and phenology of some plant species (Nautiyal et al. 2004; Khanduri et al. 2008). Despite the high ecological and economic importance of Himalayan medicinal plants, the effects of climate change on secondary metabolite production are still poorly known.

Therefore, the present study was envisaged to understand the effect of contrasting environments on biochemical and antioxidant properties of five important medicinal plants viz., *Echinacea purpurea* L., *Matricaria chamomilla* L., *Hypericum perforatum* L., *Thymus vulgaris* L. and *Cynara cardunculus* L. in Western Himalayas. All these plants are cultivated throughout the temperate regions of the world and are used for various ailments in traditional system of medicine (Table 1). The role of bioactive compounds like phenols, flavonoids, non-protein amino acids, proline, sugars and proteins, in plant adaptation to the stress environment has been thoroughly investigated in a large number of species (Guy et al. 2008). Variation in their accumulation that can have significant ecological and evolutionary implications were however, not studied at least in relation to different environments (Bidart- Bouzat and Imeh-Nathaniel 2008). The objective of present study was therefore not to find newer adaptive mechanisms for these compounds, but to assess their production dynamics in different environment in Western Himalayas, which will remain a critical feature in climate change research. This study would also open possibility of exploring the cultivation of these medicinal plants in areas other than their traditional geographical regions.

1 Methodology

1.1 Planting material

Authenticated seeds of *Hypericum*

Table 1 Native range and medicinal uses of five medicinal herbs used in the present study

Plant species (*Family*)	Native range	Medicinal use as reported in literature
Hypericum perforatum L. *(Hypericaceae)*	Europe, North Africa, Western Asia	Traditionally against anxiety, depression, cuts, and burns. Recent research shows its properties against cancer, inflammation-related disorders, and bacterial and viral diseases, and as an antioxidant and neuroprotective agent.
Matricaria chamomilla L. *(Asteraceae)*	Southern and Eastern Europe	Anti-inflammatory, sedative, antiseptic, carminatic, spasmolytic and ulcer-protecting properties.
Thymus vulgaris L. (*Lamiaceae*)	Southern Europe, Western Mediterranean region, Southern Italy	Antihelminthic, expectorant, antiseptic, antispasmodic, antimicrobial, antifungal, antioxidative, antivirotic, carminative, sedative, and diaphoretic properties
Cynara cardunculus L. *(Asteraceae)*	Mediterranean region	Hepatoprotective, anti-oxidative, anti-inflammatory, anti-microbial, anti-mutagenic and anti-proliferative properties.
Echinacea purpurea L. (*Asteraceae*)	Eastern North America	Enhance the activity of immune system, relieve pain, reduce inflammation, and have hormonal, antiviral, and antioxidant effects.

perforatum L., *Matricaria chamomilla* L., *Thymus vulgaris* L., *Cynara cardunculus* L. and *Echinacea purpurea* L. were collected from seed bank of Indian Institute of Integrative Medicine (IIIM). Details of their native range and the medicinal uses of these plants have been described in Table 1. Seeds were grown in well maintained experimental farms of IIIM at Jammu (305 m asl; 32°43'N, 74°54'E) and Srinagar (1730 m asl; 34°50'N, 74°47'E) in randomized block design with three blocks of each plant species. Due to difference in environmental conditions, the growing vegetative season varies in Jammu and Srinagar. Leaf material was therefore collected during flowering stage at 180±20 days after plantation (DAP) on bright sunny day at 10:00 hrs and immediately stored in liquid nitrogen for biochemical and antioxidant estimations. The main environmental differences in growing conditions at two locations have been presented in Table 2.

Table 2 Climatic conditions of IIIM experimental farms at Jammu and Srinagar in which five medicinal herbs (*Hypericum perforatum* L., *Matricaria chamomilla* L., *Thymus vulgaris* L., *Cynara cardunculus* L. and *Echinacea purpurea* L.) were grown.

Climate conditions		Jammu	Srinagar
Climate		Subtropical	Temperate
Altitude (meter above sea level)		305	1730
Geographic co-ordinates		32°43'N/ 74°54'E	34°50'N /74°47'E
Temperature (°C) during Flowering	Maximum	34.5 ± 1.9	27.4 ± 1.4
	Minimum	26.0 ± 2.9	12.6 ± 1.2
Relative Humidity (%)		75.6 ± 4.1	67.4 ± 2.5
Soil analysis	pH	7.28 ± 0.04	7.17 ± 0.19
	E.C. (dSm^{-1})	0.526 ± 0.034	0.522 ± 0.028
	Organic Carbon (%)	0.82 ± 0.08	1.11 ± 0.07
	Nitrogen (Kg ha^{-1})	405 ± 11.4	544 ± 18.6
	Phosphorous (Kg ha^{-1})	10 ± 1.80	13 ± 2.12
	Potassium (Kg ha^{-1})	226 ± 7.8	277 ± 2.36

1.2 Estimation of phenol and flavonoids

Total phenolic and flavonoid content was determined essentially as described earlier (Kaur et al. 2013a). Briefly, 500 mg of leaf material was extracted in 1 mL of methanol. 100 µL of methanolic extract was mixed with 100 µL of 1 N Folin–Ciocalteu reagent. Following incubation for 5 min, 200 µL of 20% Na_2CO_3 was added. Absorbance at 730 nm was measured in plate reader after 10 min and the concentration of phenolic compounds was calculated using standard curve of gallic acid (5–50 nmoles; R^2= 0.967). The results are expressed as nano moles gallic acid equivalent (nmoles GAE) per mg fresh weight of plant material. For flavonoid content, methanolic extracts were mixed with 30 µL of a 5% $NaNO_2$ solution and incubated for 5 min. 300 µL of 10% $AlCl_3.H_2O$ solution was added followed by 200 µL of 1 M NaOH and 200 µL of distilled water after 6 min. Absorbance was read at 510 nm and total flavonoids were calculated using quercetin as standard (5–50 nmoles; R^2 = 0.999). The results are expressed as nano moles quercitin equivalent (nmoles QAE) per mg fresh weight of plant material.

1.3 Glutathione and H_2O_2 estimation

Glutathione was measured essentially as described by Kaur et al. (2013b). Quantifications were done from GSH standard curve that was in the range of 0–1000 pmol. Hydrogen peroxide was estimated using Xylenol orange (XO) method (Okuda et al. 1991). 100 mg of plant tissue was crushed in 1 mL of 0.2 N $HClO_4$ and then centrifuged at 10,000 *g* for 10 min at 4°C. Supernatant was neutralized with 4 N KOH to pH 7.5 and again centrifuged for 10 min at 10,000 *g*. 1 mL XO was added to 100 µL of supernatant and incubated for 40 min at room temperature in dark before taking readings in A_{500}. Quantification was done based on standard curve made by using 100-1000 µM of H_2O_2 solution.

1.4 Ascorbate peroxidase activity

Ascorbate peroxidase activity was determined by following oxidation rate of ascorbate at 290 nm (Vyas et al. 2007). The reaction (1 mL) was performed at 25°C in 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.5 mM ascorbate and 1 mM H_2O_2. The reaction was started by addition of 10 µL of the enzyme extract in a quartz cuvette and the decrease in absorbance at 290 nm was continuously monitored for 10 min (ε of ascorbate = 2.8 mM^{-1} cm^{-1}).

1.5 Chlorophyll and xanthophyll content

Total chlorophyll was estimated in leaf material by crushing 100 mg of tissue in acetone and read at absorbance 645nm and 663nm. Concentration of chlorophyll was calculated using the formulae.

Total Chlorophyll = $(A_{645}\times20.2) + (A_{663}\times8.02)$

Chlorophyll A = $(A_{663}\times12.7) - (A_{645}\times2.69)$

Chlorophyll B = $(A_{645}\times22.9) - (A_{663}\times4.68)$

For xanthophyll estimation, 100 mg of plant samples was crushed with 1 mL of cold acetone and volume of extract was increased to 10 mL with acetone. This mixture was then kept in dark for 30 minutes and centrifuged at 9600 *g* for 10 min. Quantitative estimation of xanthophylls was performed by measuring the absorption A_{445} of acetone extracts using E = 2340 (Deineka et al. 2007).

1.6 Other biochemical estimations

Primary metabolites like proteins, reducing sugars and free amino acid content were measured using standard procedures as described by Sawhney and Singh (2009). Protein content in leaf tissue was calculated following the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. Reducing sugar content was analyzed following the method by Zhu et al. (2005) using glucose as standard.

For free amino acids, 100 mg of plant material was crushed in 10 mL of 70% ethanol and centrifuged at 2700 *g* for 10 min. Supernatant was collected and pellet was re-extracted in 70% ethanol. Combined ethanolic extract was dried on a boiling water bath and 0.2 M citrate buffer was added to the pellet. Half the volume of KCN-acetone ninhydrin solution was added to the citric buffered preparations and incubated in water bath for 20 min. The solution was cooled and A_{570} was recorded in a spectrophotometer. KCN-acetone ninhydrin solution oxidizes all the amino acid and gives a purple colored complex, which is quantified using standard curve of glycine prepared in the range of 10 – 100 mg.

Proline content was measured according to the method of Bates et al. (1973). Briefly, 100 mg of plant material was crushed in 1 mL of 3% sulphosalicilic acid. Then, the homogenate was centrifuged at 9600 *g* for 10 min at 4°C. To 20 µL of supernatant, acid ninhydrin was added and incubated at 100°C for an hour. After incubation, absorbance was taken at 520 nm.

1.7 Statistical analysis

Each value for the biochemical determination represents the average of at least three biological repeats one from each block (± standard deviation). Statistical analysis was performed using IBM® SPSS® Statistics version 20.0 program on each experiment using t-test and values of $P \leq 0.05$ were considered to be significant.

2 Results and Discussions

Fluctuating atmospheric conditions have now become rule rather than exception due to climate change scenario, more so in Himalayan biodiversity region. Precipitation and temperature variations affect the chemical composition and, ultimately the survival of some medicinal plants in high altitude region. Particularly, the temperature stress can affect secondary metabolites and other compounds that plants produce, which are usually the basis for their medicinal activity (Zobayed et al. 2005; Salick et al. 2009). The present study therefore is an attempt to understand the accumulation of bioactive compounds at two contrasting locations in Western Himalayas.

Both phenols and flavonoids (Figure 1) were found significantly higher in plants growing at temperate climate of Srinagar. These compounds are commonly found in plant kingdom that actively participate as reducing and scavenging agents, thereby suggesting that the plants grown at higher altitudes will have increased medicinal properties. The redox properties of phenolic compounds allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Soobrattee et al. 2005). Most prominent difference was found in *E. purpurea* where, values of phenols were 3.73 times higher at Srinagar than Jammu. *T. vulgaris* did not show significant change in phenol content at the two locations. Among species, maximum phenolic content was observed in *T. vulgaris* (76.33 nmol mg^{-1} FW) and *H. perforatum* (70.33 nmol mg^{-1} FW)

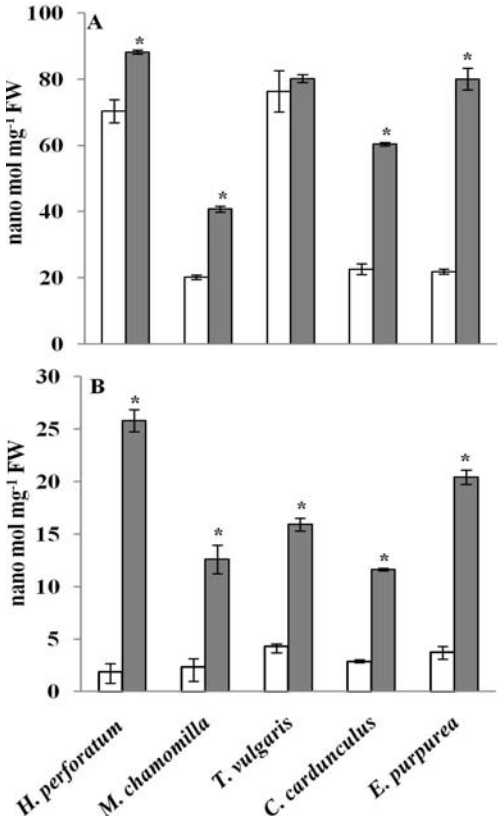

Figure 1 Variations in total phenols (A) and total flavonoids content (B) in *Hypericum perforatum* L., *Matricaria chamomilla* L., *Thymus vulgaris* L., *Cynara cardunculus* L. and *Echinacea purpurea* L. at Jammu (□) and Srinagar (■). * represents statistically significant values between the two altitudes at a *P* value lower than 0.05.

at lower altitude, whereas, at higher altitude the phenolic content was found to be maximum in *H. perforatum* followed by *T. vulgaris* and *E. purpurea*. The percentage change in flavonoids from Jammu to Srinagar in different plants ranged from 3.69 in *T. vulgaris* to 13.91 in *H. perforatum*. Thus, *H. perforatum* was found to be the species containing highest content of phenols and flavonoids at least when grown in high altitude temperate environment. Flavonoids are widespread plant secondary metabolites, including flavones, flavanols, and condensed tannins. The free radical scavenging activity of flavonoids is dependent on the presence of free OH groups especially 3-OH. It has been reported that plant flavonoids that show antioxidant activity in vitro also function as antioxidants in vivo (Geetha et al. 2003; Agati et al. 2012). Our study suggested that temperate high altitude climate also induces the metabolic content of phenols and flavonoids probably as a strategy to cope with increased light. Plant phenolics have been shown as a defence strategy during increased light intensity and UV proportion of the light (Vergeer et al. 1995; Khandaker et al. 2010; Bravo et al. 2012), which is a characteristic feature of high altitudes (Körner 2007).

Another factor that might play a crucial role in decreased metabolic content of these phenols and flavonoids at lower sub-tropical environment could be higher temperature. As observed in Table 2, the maximum and minimum temperatures in Jammu are 7°C to 13 °C higher than Srinagar during flowering phase (when the samples were collected for analysis). Any increase in the temperature would hence deteriorate the metabolic content of bioactive compounds, which is likely to be the scenario during climate change (Snow et al. 2003). It has been suggested that increase in temperature would be an essential response of climate change especially in Western Himalayas (Miller-Rushing and Primack 2008; Bhutiyani et al. 2007). Challenges posed by climate change could push some important species to extinction and may result in decrease in number of endemic species in the region as species composition, structure and functioning of sensitive habitats can change both because of increased temperatures (Gairola et al. 2010).

The inherent capacity of individual plants in tolerating the stress at any location can be judged by its metabolic content of ROS (Foyer and Noctor 2005). In this study, when the H_2O_2 content was measured in all the five species, *M. chamomilla and C. cardunculus* showed 3.12 and 2.01 times higher concentration in Srinagar than Jammu, whereas, H_2O_2 content was found lower in *H. perforatum* (135.22 μmol mg^{-1} FW) and *T. vulgaris* (175.04 μmol mg^{-1} FW). No significant change was observed in *E. purpurea* at both locations. Lower content of H_2O_2 even in putative stressful environment suggested better antioxidant machinery in *H. perforatum* (Figure 2A). As discussed earlier, one of the reasons for this could the higher content of phenols and flavonoids (Figure 1). Chloroplasts have been the source of H_2O_2 production at higher altitudes largely due to the slippage of electrons during photosynthesis (Vyas et al. 2007). Ascorbate peroxidase (APX) have shown higher affinity towards H_2O_2

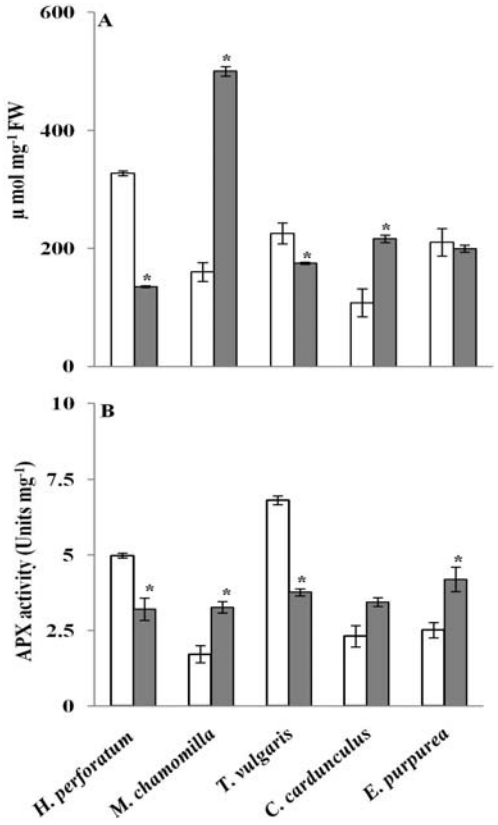

Figure 2 Metabolic content of H_2O_2 (A) and APX activity (B) in *Hypericum perforatum* L., *Matricaria chamomilla* L., *Thymus vulgaris* L., *Cynara cardunculus* L. and *Echinacea purpurea* L. at Jammu (□) and Srinagar (■). 1 unit of enzyme activity was defined as mmol of ascorbate oxidized mg^{-1} fresh weight min^{-1}. *represents statistically significant values between the two altitudes at a *P* value lower than 0.05.

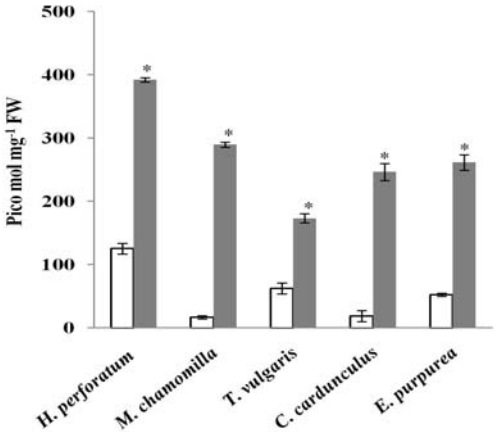

Figure 3 Metabolic content of total glutathione in *Hypericum perforatum* L., *Matricaria chamomilla* L., *Thymus vulgaris* L., *Cynara cardunculus* L. and *Echinacea purpurea* L. at Jammu (□) and Srinagar (■). * represents statistically significant values between the two altitudes at a *P* value lower than 0.05.

scavenging and is localized in chloroplast (Ishikawa and Shigeoka 2008), hence APX activity was estimated to assess the scavenging capacity of H_2O_2 at both the locations (Figure 2B). Variation in the APX activity was observed at Jammu with *T. vulgaris* showing highest activity, whereas, the enzyme activity is almost similar in all plant species at Srinagar. This suggests that the enzymatic scavenging potential is overwhelmed and other potential scavenging metabolites such as phenols and flavonoids also play important role in ROS scavenging. Also, intracellular ROS can interact with reduced glutathione and helps in maintaining the redox status of the cell (Noctor et al. 2012). It is therefore observed that glutathione status is a useful marker for oxidative stress triggered by increased intracellular H_2O_2 production. When glutathione was observed in plants at both the locations, it was observed that plants at Srinagar had significantly higher content of total glutathione than plants growing at Jammu (Figure 3). It is possible largely due to the increase in the reduced form followed by the induction of genes encoding enzymes involved in cysteine synthesis in the chloroplast (Queval et al. 2009). As far as variations in the individual plants are concerned at the two locations, it could also be the differences in the basal level of the metabolites and anti oxidative enzymes. Similar, differential metabolite content was observed in studies of various other medicinal plants (Rao et al. 2012; Jan et al. 2014).

Results of photosynthetic pigments reveal that the content of total chlorophyll (Figure 4A) was lower to nearly half amount in plants at Srinagar than in Jammu. It suggests the role of high light in reducing the chlorophyll content. Several studies have reported the similar pattern in various plants (Zhang et al. 2005). Ratio of Chl a/b (Figure 4B) as observed in various plants (Walters 2005; Portes et al. 2010) suggest the modulation in PSII photosynthetic machinery in plants at higher altitudes. Smaller antennae size suggested by lower Chl a/b ratio would not only mitigate efficiency losses associated with non photochemical quenching but also allow a greater transmittance of light into lower layers of the canopy or cells towards the lower surface of the leaf (Melis et al. 1998). Xanthophylls however showed higher values in plants at Srinagar than Jammu (Figure 4C). The

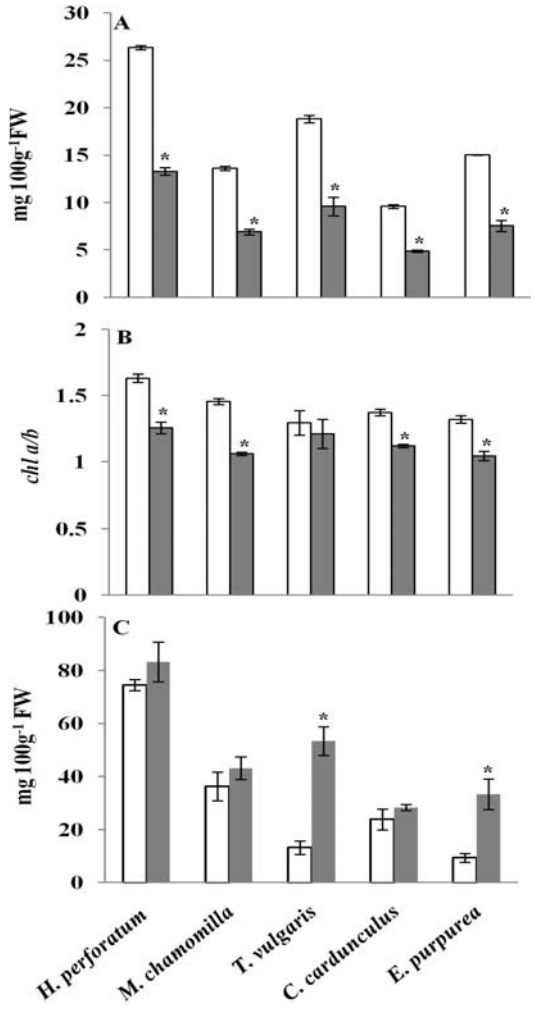

Figure 4 Content of total chlorophyll (A), chlorophyll a/b ratio (B) and xanthophyll (C) in *Hypericum perforatum* L., *Matricaria chamomilla* L., *Thymus vulgaris* L., *Cynara cardunculus* L. and *Echinacea purpurea* L. at Jammu (□) and Srinagar (■). * represents statistically significant values between the two altitudes at a *P* value lower than 0.05.

role of xanthophylls in the protection of photosynthesis in plants has been widely reviewed

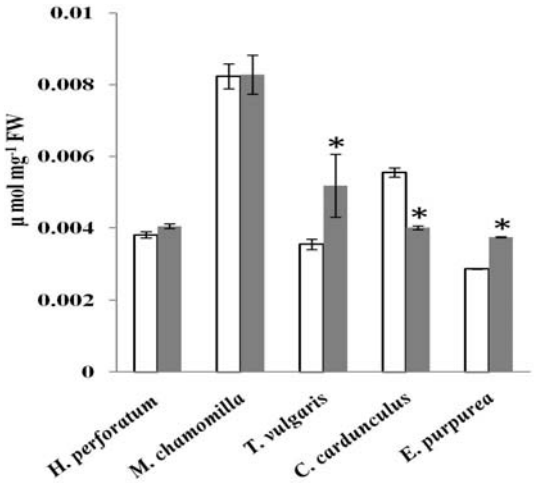

Figure 5 Variations in accumulation of proline content in *Hypericum perforatum* L., *Matricaria chamomilla* L., *Thymus vulgaris* L., *Cynara cardunculus* L. and *Echinacea purpurea* L. at Jammu (□) and Srinagar (■). * represents statistically significant values between the two altitudes at a *P* value lower than 0.05.

earlier (Demmig-Adams and Adams 1996; Jahns et al. 2012). Thus, it is suggested that chlorophyll content, chlorophyll structure and xanthophyll pigments play a crucial role protecting photosynthetic apparatus in plants at higher altitude temperate climate.

Protection to various stresses is not only dependent on the higher amount of antioxidant compounds, but role of primary metabolites such as soluble proteins, reducing sugars and free amino acids has also been found to be important (Jan et al. 2014). Total crude protein and reducing sugars were found to be higher in all the plants growing at Srinagar (Table 3). Induction in the activities of anti-oxidant enzymes could be one of the strong reasons for this accumulation. Earlier, we have found induction in antioxidant enzymes in high altitude

Table 3 Effect of two cultivation sites on content of total protein (µg 100 mg^{-1} FW), reducing sugars (µg 100 mg^{-1} FW) and free amino acid (µg 100 mg^{-1} FW) in *Hypericum perforatum* L., *Matricaria chamomilla* L., *Thymus vulgaris* L., *Cynara cardunculus* L. and *Echinacea purpurea* L. at Jammu (305 m) and Srinagar (1730 m). * represents statistically significant values between two sites at a *P* value lower than 0.05.

	Total protein		Reducing sugars		Free amino acid	
	Jammu	Srinagar	Jammu	Srinagar	Jammu	Srinagar
Hypericum perforatum	4244 ±257	6411 ±93.0*	237.03 ±8.98	642.90 ±18.1*	4.94 ±0.29	6.07 ±0.14*
Matricaria chamomilla	2909 ±213	3798 ±124*	150.37 ±6.41	625.25 ±36.1*	7.77 ±0.75	8.91 ±0.17*
Thymus vulgaris	2707 ±149	4969 ±3.95*	202.22 ±3.84	624.44 ±8.85*	3.51 ±0.30	4.08 ±0.03*
Cynara cardunculus	3762 ±159	6248 ±15.9*	185.18 ±18.8	605.33 ±68.8*	1.67 ±0.10	1.75 ±0.10
Echinacea purpurea	3203 ±164	4489 ±15.9*	139.25 ±4.62	395.03 ±17.4*	2.56 ±0.40	3.05 ±0.16

grown *Lepidium latifolium* L. (Kaur et al. 2013b). Sugars and carbohydrates have been used as a strategy to accumulate reserves under non-optimal photosynthetic conditions or low temperatures (Lütz 2010; Nägele and Heyer 2013). Free amino acids and proline were shown to have specific role in osmoregulation (Dedemo et al. 2013; Rai 2002) and plant defense (Forde and Roberts 2014) and exhibited differential accumulation at both the locations (Table 3, Figure 5).

3 Conclusions

The present study therefore concludes that species specific changes are observed in biochemical and antioxidant profiling of five medicinal plants at two climates. Although, stressful environment was observed in higher altitudes of temperate climate, plants have adapted themselves by accumulation of metabolites that harbor tolerance. When these plants were grown in environment different (subtropical) from their native (temperate), they show lesser amount bioactive compounds like phenols and flavonoids. There are certain species specific variations in some primary metabolites like proline and xanthophyll, however, these do not have direct influence on the medicinal properties. There appears little potential for expanding the geographical cultivation area of these plants. Efforts should therefore, be focused to address the problem of climate change that otherwise can have significant ecological and evolutionary implications for these high value medicinal plants in Western Himalayas owing to variations in induction of plant bio-chemicals.

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