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Research Article

Altitudinal variation in some phytochemical constituents and stomatal traits of *Primula denticulata*

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Abstract

Due to extensive medicinal importance and wide distributional range of *Primula denticulata* Sm., locally known as Doker-neej (Kashmiri) of family Primulaceae, in Kashmir valley, the present investigation was carried out to study the variations in phenology, stomatal traits and content of phytochemicals of the species in relation to altitude. During the present investigation, trends in the stomatal traits which include: stomatal density, length and breadth of guard cells and potential conductance index (PCI) along an altitudinal gradient were analyzed by using standard methods. Samples from different populations with varying altitudes were also subjected to qualitative and quantitative estimation for the presence of various phytochemicals by using various spectrophotometric techniques. It was observed that mean values of the analyzed stomatal traits in the four populations varied significantly ($p \leq 0.05$) across the populations. Phytochemicals such as phenols, tannins, alkaloids and saponins were present in all the sampled populations but their quantity varied significantly across the populations. This assessing of phytochemical content of plants at varying altitudes can help to select elite genotype and reflect the best suited altitude for commercial cultivation of the species as these phytochemicals are considered as the basis for their medicinal activity.

Keywords: *Primula denticulata*, phytochemicals, stomatal traits

1. Introduction

Biosynthesis of the secondary metabolites in plants is not only controlled genetically, but is also strongly affected by different biotic and abiotic stresses[1]. In different ecological niches, plants behave differently in terms of biochemical aspects in order to better adapt to their environment. This broad range of environmental factors across altitude thus affects the chemical composition and, ultimately the survival of medicinal plants in such regions. Thus the stress conditions affects secondary metabolites or so called active ingredients and other compounds that plants produce, which are usually the basis for their medicinal activity[2]. Plants at various altitudes can adapt by avoiding and overcoming the stress conditions by means of various physiological and biochemical mechanism including evolution of a resistance-conferring genotype, or by improvement of genes which can produce ecologically adapted phenotypes or can have a different response related to their resistance to these stress conditions which depends mainly on the morphology, anatomy and life cycle[3].

Primula denticulata Sm., distributed in the mountain ranges of Kashmir Himalaya is confined to temperate, sub-alpine and alpine regions, grows in early spring in forests, near melting glaciers, moist meadows and grassy slopes, ranging in altitude from 2100 – 4050m asl indicating that the species can tolerate a broad range of environmental conditions. The species grows wild in Kashmir Himalaya and bear remarkable medicinal importance[4]. The chemical profiling of *Primula denticulata* (Indian anti-snake venom plant) showed presence of Primetin-19 (5, 8-dihydoxyflavone) which posses strong sensitizing properties and is a powerful contact allergen[5].

Due to extensive medicinal importance and wide distributional range of *P. denticulata* in Kashmir valley, the present investigation was carried out to study the variations in its Phenology, anatomy

and content of phytochemicals with altitudes, as every hundred feet of elevation brings some new phase of climate and vegetation in Kashmir Himalayas[6]. Also much little work has been done concerning this facet in Kashmir valley. This sampling of populations from habitats with varying altitudes can allow us to assess the intraspecific variations and main ecological trends of phytochemical accumulation in plants.

2. Material and methods

2.1. Study sites

Primula denticulata was collected from five different sites with varying altitudes in Kashmir valley. The detailed geographic features of these study sites are given in table 1.

Table 1: Geographical coordinates of the selected sites

S. No	Study Site	District	Altitude (m asl)	Geographical Co-ordinates	Habitat
1.	Apharwat	Baramulla	2850	34° .03′141″N 74° 35′154″E	Moist slopes
2.	Gulmarg	Baramulla	2500	34°·3′141″N 74° 35′154″E	Grassy Slopes
3.	Naranag	Ganderbal	2400	34°·21′370″N 74° 58′900″E	Moist slopes
4.	Tangmarg	Baramulla	2250	34°·07′890″N 74° 50′700″E	Moist slopes
5.	KUBG*	Srinagar	1595	34° 30´990″N 75° 30´520″ E	Sandy loam

*KUBG: Kashmir University Botanical Garden

2.2. Phenology

Different phenophases such as vegetative phase, flowering phase and senescence were recorded from the tagged individuals in each population throughout the growing season. The data were recorded over a period of two growing seasons (2011 to 2012).

2.3. Stomatal traits

Plant material was collected from four different populations across Kashmir valley for studying stomatal characteristics and anatomical features.

2.4. Stomatal density (count/mm^2) and stomatal parameters

Stomatal density was estimated by collecting leaf samples from 5-6 individuals of the species at each selected site and was immediately fixed in FAA (5ml Formalin: 5ml Acetic acid: 90ml 70% Ethyl alcohol). After clearing the piece of the leaf (middle part), upper and lower epidermis was peeled out separately by means of forceps and mounted in glycerin water on a slide. Then area of 1mm^2 was scanned under microscope and the number of stomata present in the area was counted[7] Stomatal parameters like guard cell lengths and breadth of 10 stomata were measured at a magnification of 40X from each of the leaves from selected populations. Potential conductance index (PCI = guard cell length^2 × Stomatal density × 10^{-4}) was also determined.

2.5. Stem anatomy

Plant parts were first fixed in FAA. The wax-embedding procedure[8] was followed for studying the anatomy of leaf. Section cutting of stem and leaf were carried out by using Microtome and 7µm thickness sections were cut and these sections were subsequently stained with safranin and crystal violet. The cross sections were passed through different alcohol grades and were subsequently studied under light microscope (10X) and photographs were taken with the help of Trinacular microscope at USIC, Kashmir University.

2.6. Plant material collection

Healthy and disease free plants of *Primula denticulata* were collected from different study sites with varying altitudes at the time of flowering stage in order to avoid variations due to different developmental stages. The specimens were identified and deposited in Kashmir University Herbarium (KASH) under voucher no KASH-1743 and KASH-1744.

2.7. Qualitative screening of phytochemicals

Phytochemical screening for major bioactive constituents like phenolics, flavonoids, tannins, alkaloids and saponins was undertaken using standard phytochemical methods[9].

2.8. Quantitative screening

2.8.1. Phenolics determination

Total phenolic content was determined by Folin–Ciocalteu method[10] with slight modifications. To 1g of sample powder, 2.5ml of ethanol was added and centrifuged at 2°C for 10min. Supernatant was evaporated to dryness and redissolved in 10ml water. 200µl of sample was added to 100µl diluted (1:10) Folin–Ciocalteu reagent and equilibrated for few min. Then 800µl of 2.5 % aqueous Na_2CO_3 was added and mixture was allowed to stand for 60min at room temperature

with intermittent shaking. The absorbance of the blue colour solution was measured at 765nm on UV–visible spectrophotometer. Gallic acid (50mg %) was used as standard. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE mg/g dry weight of sample) and the values were presented as mean ±SD of triplicate analysis.

2.8.2. Alkaloid determination

2.5g of the powder was extracted using 100ml of 20% acetic acid in ethanol. The solution was covered for almost 4 hours. Filtrate was concentrated to 25ml. Concentrated ammonium hydroxide was added stepwise to attain precipitation. The whole solution was kept as such until precipitate settled. Collected precipitate was washed with dilute ammonium hydroxide and finally filtered. Filtrate was discarded and pellet obtained was dried and weighed[11] and the values were presented as mean ± SD of triplicate analysis.

2.8.3. Saponin determination

10g of sample was mixed with 100ml of 20% aqueous ethanol. The mixture was kept for 4hr on water bath shaker at 55°C. The extract was concentrated to 40ml over water bath at 90°C. Concentrate obtained was transferred into a separating funnel and 10ml of diethyl ether was added to it. After shaking vigorously aqueous layer was recovered and ether layer was discarded. The process was repeated. To the aqueous layer n-butanol was added. The whole mixture was washed in separating funnel twice with 10ml 5% of aqueous NaCl. Upper part was retained and heated in water bath until evaporation. Latter it was dried in oven to a constant weight[12] and the values were presented as mean ± SD of triplicate analysis.

2.8.4. Tannins determination

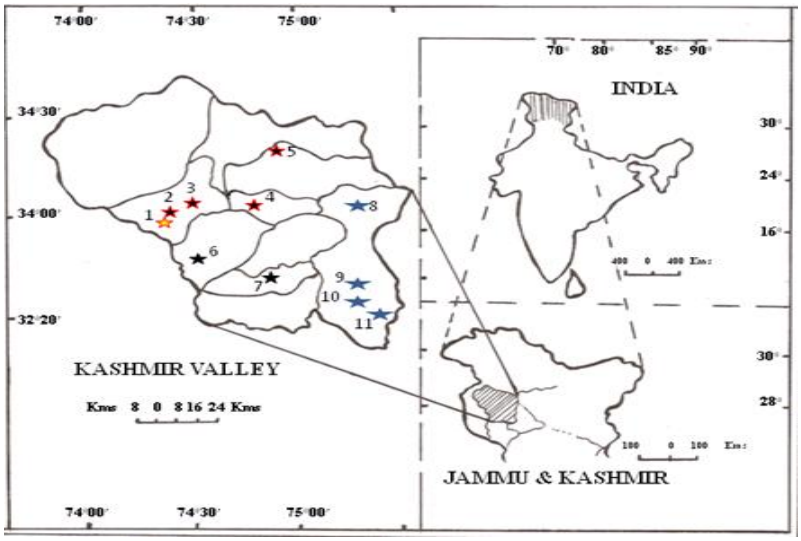
2g of plant powder was extracted thrice in 70% acetone. After centrifuging the sample supernatant was removed. Different aliquots were taken and final volume to 3 ml was adjusted by distilled water. The solution after vortexing were mixed with 1 ml of 0.016M $K_3[Fe(CN)_6]$, followed by 1ml of 0.02M $FeCl_3$ in 0.10M HCl. Vortexing was repeated and the tubes were kept as such for 15min. 5ml of stabilizer (3:1:1 ratio of water, H_3PO_4 and 1% gum Arabic respectively) was added followed by revortexing. Absorbance was measured at 700nm against blank. Standard curve was plotted using various concentrations of 0.001M gallic acid[13].

3. Results

3.1. Distribution

Extensive field survey revealed that the species grows in Kangdoori, Doodhpathri, Gulmarg, Tangmarg,, Shopian, Chandanwari, Morgan top, Simpthon top, Daksum, Yusmarg and Naranag areas of J&K state (Fig. 1). Of these; four sites of varying altitudes viz.; Apharwat, Gulmarg, Tangmarg, Naranag**,** and one population at KUBG (Kashmir University Botanical Garden)**,** were selected for the present investigation. The salient features of the selected sites are summarized in Table 1.

Fig. 1: Map showing the study areas



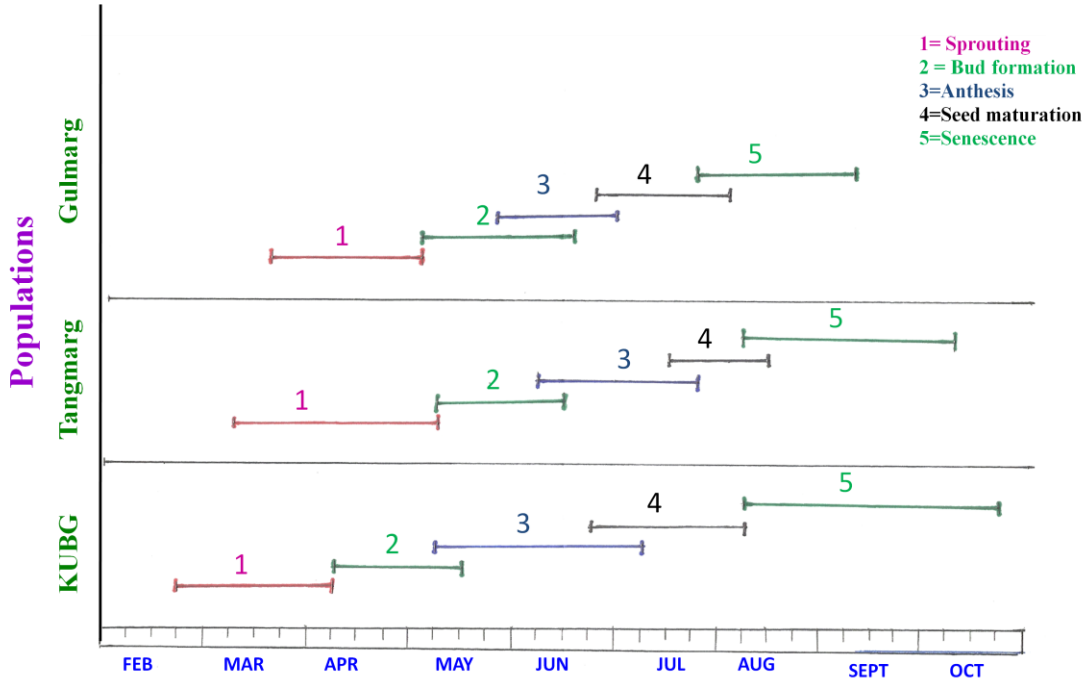
1.Kangdoori; **2.**Gulmarg; **3.**Tangmarg; **4**.Naranag; **5**.KUBG; **6**.Doodpathri; **7**.Shopian; **8**.Chandenwari; **9**.Margan; 1**0**.Daksum

3.2. Phenology

The phenological behavior of the species was monitored in the selected populations at three different altitudes of Kashmir valley. During the present investigation, it was observed that life span of the species goes on decreasing as altitude increases

and plants at higher altitudes try to complete their life cycle quicker than low altitude ones (Fig. 2).

Fig. 2: Phenogram of *Primula denticulata* at different altitude



3.3. Changes in stomatal traits with altitude

Stomatal size and density are considered two key ecophysiological parameters, as they influence stomatal conductance. Leaf samples were collected from three sites with varying altitude i.e., Apharwat (2850m asl), Gulmarg (2500m asl) Tangmarg (2250m asl) and one population growing at the Kashmir University Botanical Garden (KUBG) with an altitude of 1595m asl. During the present study, trends in the stomatal traits were analyzed which include: stomatal density, length and breadth of guard cells and potential conductance index (PCI) along an altitudinal gradient and it was observed that mean values of the analyzed stomatal traits in the four populations varied significantly ($p \leq 0.05$) across the populations (table 2). Among selected populations the leaves from Apharwat population possesses highest stomatal density (70.50±0.88) as compared to other three selected populations. Guard cell length also varied across the populations with Apharwat possessing longest guard cell length (37.56±1.086µm) as compared to other three populations. A linear correlation between altitude and guard cell length was observed in the species ($r = 0.65$, $p \leq 0.05$). Guard cell breadth of the species decreases with increase in altitude therefore showing a negative correlation between altitude and guard cell breadth. Potential conductance index depends on both stomatal density and size of stomatal aperture. PCI in the species increased with increase in altitude and was found to be significantly correlated ($r = 0.95$, $p \leq 0.05$) with altitude (Fig 3).

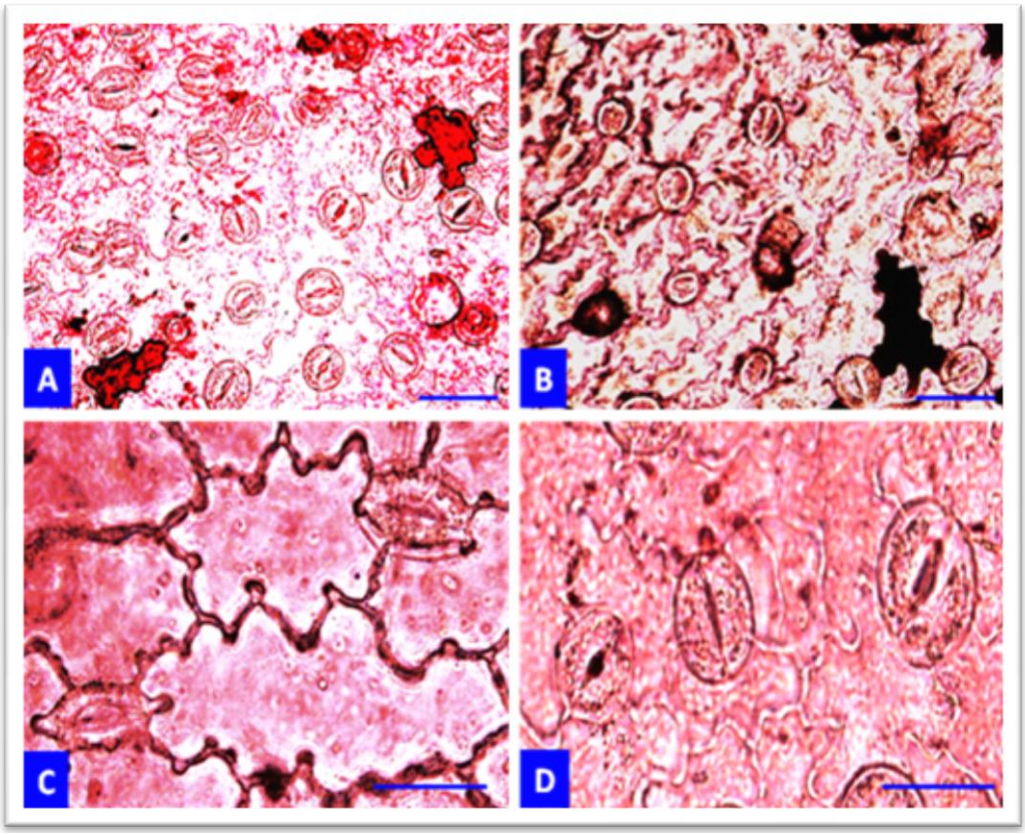
All the four analysed populations showed presence of trichomes but the number and size of these trichomes increased with altitude. The trichomes were dense at high altitude (Gulmarg-I) and sparse at low altitude (KUBG) (Fig. 4). Also the plants growing at high altitudes particularly near melting glaciers with moist soil show presence of aerenchyma in cortex and pith of stem while the plants growing at lower altitude with dry soil does not show the presence of aerenchyma in cortex and pith of the stem (Fig. 5).

Table 2: Variation in various stomatal traits of *P. denticulata* across different populations and their correlation with altitude

Trait	Population						
	Apharwat 2850m asl	Gulmarg 2500m asl	Tangmarg 2250m asl	KUBG 1595m asl	P	F	Correlation (r)
Stomatal density (count/mm^2)	70.50±0.8*	67.166±1.7	56.50±1.2	50.33±1.6	0.04	54.11	0.727
Guard cell length (µm)	37.56±1.08	33.51±0.22	35.07±2.6	24.68±2.2	0.21	69.9	0.65
Guard cell breadth (µm)	16.47±2.17	17.53±3.34	18.77±0.2	23.04±0.1	0.00	113.2	-0.48
Potential conductance index (PCI)	6.54±1.32	5.65±1.21	5.59±1.29	4.30±1.12	0.02	5.119	0.85

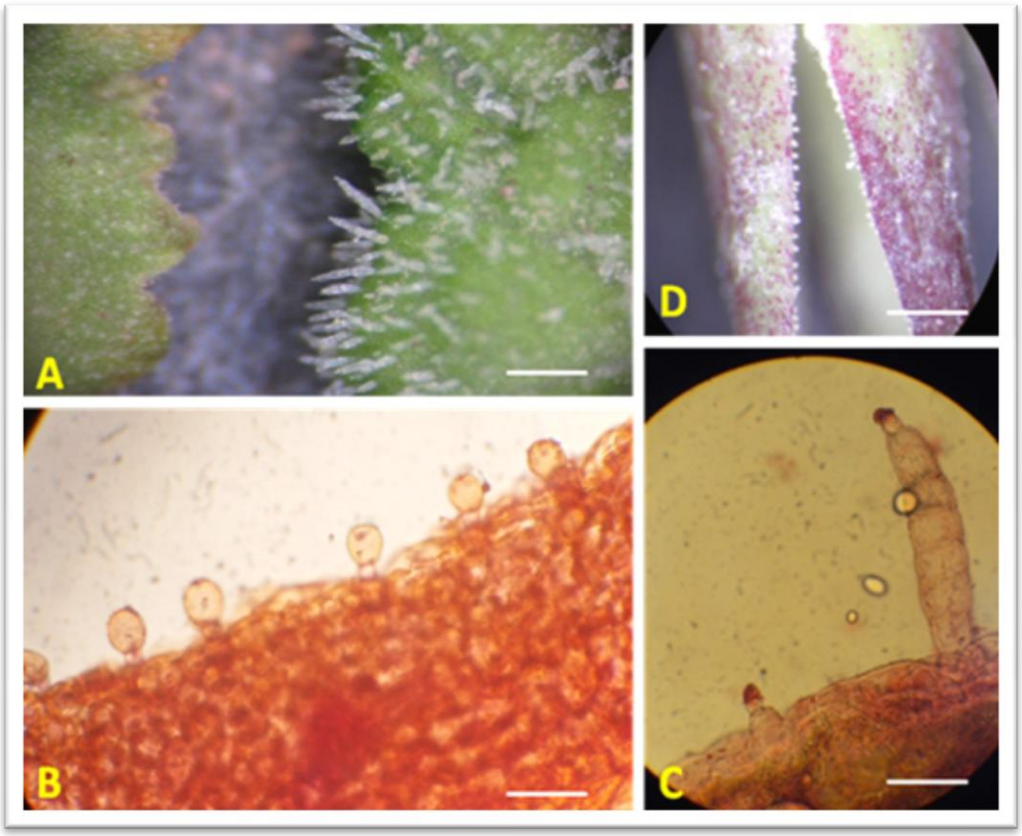
*Values are in mean ± SE

Fig. 3: Different stomatal traits of *P. denticulata*



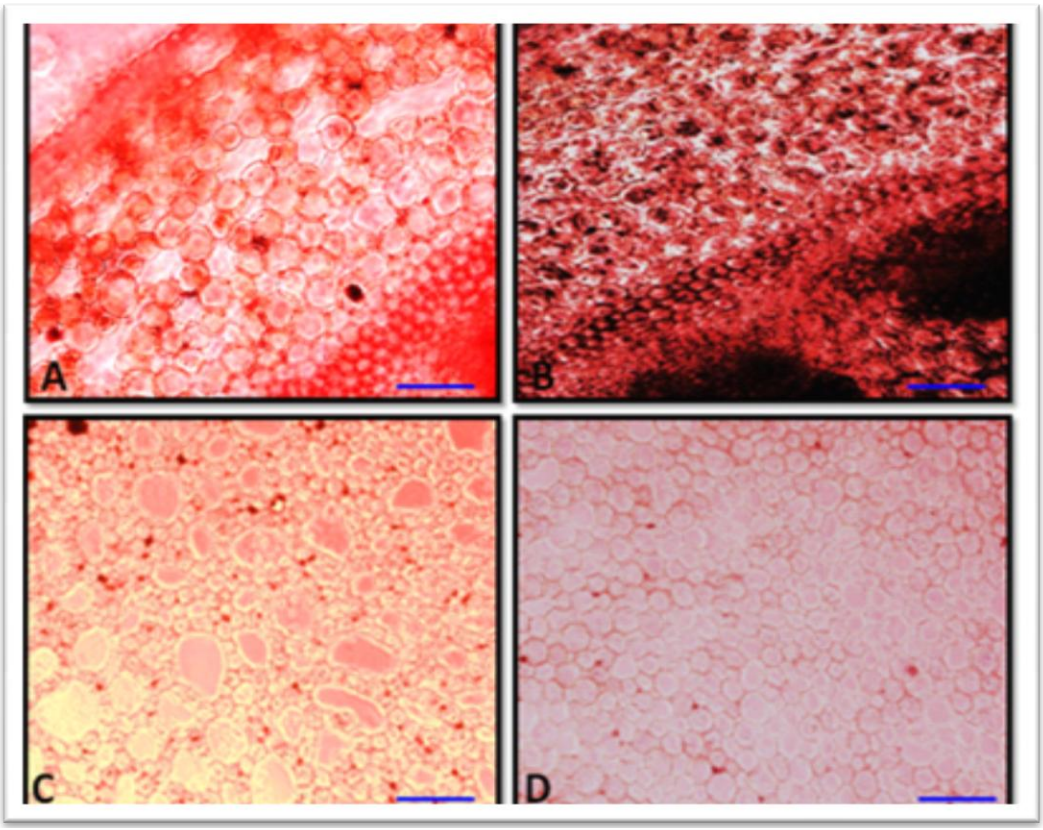
A and **B**: Stomatal density at Gulmarg and KUBG (10X); **C** and **D:** Stomatal length and breadth at Gulmarg and KUBG (40X) Scale = 10µm

Fig. 4: Trichome distribution and morphology



A and D: Comparison of trichome morphology in *P. denticulata* at low and high altitude populations (Scale=20cm); B,C and D: Muticelled trichomes of *P. denticulata* at high altitude populations (Scale=10µm

Fig. 5: Stem anatomical features



A and C: Presence of aerenchyma in stem cortex and pith of high Altitude moist Population; B and D: KUBG population without aerenchyma (Scale=10µm)

3.4. Comparative estimation of phytochemicals at different altitudes

Phytochemicals such as phenols, flovonoids, tannins, alkaloids and saponins were present in all the sampled populations but their quantity varied significantly across these populations. Quantitative estimation of phytochemicals was performed by various standard methods. It was observed during the present study that the quantity of phytochemicals increased with increase in altitude of the studied populations. Low quantity of phytochemicals was observed at KUBG (1595m asl) and highest at Apharwat (2850m asl) population table 3 and table 4.

Table 3: Qualitative phytochemical screening of *P. denticulata* from four different populations with varying altitudes

Population	Phytochemical screening				
	Phenols	Flavonoids	Tannins	Alkaloids	Saponins
Apharwat (2850m asl)	++	++	++	++	++
ApharwatI (2500m asl)	+	+	+	++	+
Naranag (2400m asl)	+	+	+	++	+
Tangmarg (2250m asl)	+	+	+	+	+

++ means abundant; + denotes average

Table 4: Comparison of different phytochemical constituents across various populations with varying altitudes

Phytochemicals (g%)	Populations/ (altitude)					ANNOVA		Correlation (r)
	Apharwat/ (2850m asl)	Gulmarg/ (2500m asl)	Naranag/ (2400m asl)	Tangmarg/ (2250m asl)	KUBG/ (1595m asl)	F	P	
Phenols	1.12±0.047*	0.94±0.019	1.33±0.02	0.81±0.023	0.34±0.031	102.9	0.02	0.850
Tannins	0.813±0.07	0.69±0.021	0.496±0.14	0.49±0.121	0.206±0.019	151.7	0.00	0.96
Alkaloids	1.72 ±0.091	1.60±0.034	1.38±0.02	1.20±0.094	0.588±0.028	82.00	0.04	0.954
Saponins	0.37±0.018	0.148±0.009	0.473±0.05	0.128±0.065	0.075±0.005	102.9	0.13	0.831

*Values are mean ± SE

4. Discussion

During the present investigation, it was observed that life span of the species goes on decreasing as altitude increases and plants at higher altitudes try to complete their life cycle quicker than low altitude ones. Our results are in congruence with results observed in *Nothophagus pumilio* of montane forests of Argentina, that bud burst and flowering both were delayed at higher altitudes as compared to low altitude ones[14]. The phenological cycle of plants is influenced to the greatest extent by temperature, photoperiod and precipitation[15] which change with increasing altitude. Stomatal parameters are specific for a particular species but are affected by multiple ecological factors, including altitudinal gradient[16]. Present study revealed a positive correlation between stomatal density and altitude. The results are in conformity with the reports observed in *Abies balsamea* where stomatal density tended to increase at higher elevations[17]. Qiang reported that stomatal density is negatively correlated with atmospheric CO_2 and attributed decreasing level of CO_2 with increasing elevation as the possible reason for increased stomatal densities[18]. Lockheart reported that CO_2 level directly affects stomatal differentiation. Guard cell breadth of *P. denticulata* decreases with increase in altitude[19]. Our results are in agreement with the results of Rafet, who also reported decrease in stomatal width in some apple cultivars at higher altitudes[20]. Guard cell length and potential conductance index generally increases with increase in altitude. There was a significant linear correlation between altitude and guard cell length in *P. denticulata.* The results of Tiwari are in congruence with our results who observed that in *Pinus roxburghii* potential conductance index and guard cell lengths were affected by environmental factors and recorded a direct correlation of PCI and guard cell length with altitude[21].

All the four analyzed populations showed presence of trichomes. The trichome density was greater at high altitude population (Apharwat) and sparse at low altitude (KUBG). Leaf trichomes are considered to protect living cells from damage and increase resistance to abiotic stress like solar UV-radiation and low temperatures, prevailing at high altitudes. Also present study revealed that the plants growing at high altitudes particularly near melting glaciers with moist soil show presence of aerenchyma in cortex and pith of stem while the plants growing at lower altitude with dry soil do not show the presence of aerenchyma. The root and stem become permeated with tissues having large intercellular spaces in waterlogged soils[22]. Armstrong considered the presence of large intercellular spaces in the tissues for ventilation and as a factor for flood tolerance in such environmental conditions[23].

Present study revealed that crude extract of *P. denticulata* contains various secondary metabolites. These phytochemicals serve as a major source for pharmaceutical products, so the plant species can hold an immense potential to serve as therapy for various chronic diseases. Highest content of phytochemicals (phenols, tannins and alkaloids) was observed in the plants collected from Apharwat with an altitude of 2850m asl. This increase in the phenolic content with increase in altitude may be ascribed as a response of plants to enhanced UV-B radiation and decreased temperatures which elicit amplified biosynthesis of UV-absorbing and antioxidant phenolics in plant[24]. Copaja reported that saponin production is higher at high altitudes where the prevailing environmental conditions are stressful. The author attributed the high production of saponins to adaptation of the plant to survive in the adverse environmental conditions[25]. The production of phenolic and saponin content is higher at Naranag population (2400m asl) which is located at low altitude than Gulmarg. The high production of these phytochemicals may be attributed to fluctuation in temperature and non availability of nutrients.

Present investigation revealed alkaloid and tannin content increases with increase in altitude, as stress conditions induce polyamine formation which results in nitric oxide biosynthesis that moves freely through the cells acting as potential chemical elicitor of alkaloid production[26]. Mossi, also suggest the existence of a correlation between environmental factors such as average annual temperature, climate, vegetation, geomorphology, latitude and altitude and tannin production[27]. Tannins are considered as the most important antioxidants against free radicals generated by various types of stress prevailing at higher altitudes[28]. Thus these climatic change across altitude could affect the chemical composition and ultimately the survival of some medicinal plants in high altitude regions as the stress particularly the temperature stress can affect secondary metabolites and other compounds that plants produce, which usually are the basis of their medicinal activity[29].

5. Conclusion

This sampling of populations from habitats with varying altitudes allows assessing the

intraspecific variations and main ecological trends of phytochemical accumulation in plants. So assessing phytochemical content of plants at varying altitudes can help to select elite genotype and reflect the best suited altitude for commercial cultivation of the species as these phytochemicals are considered as the basis for their medicinal activity. Also studies on anatomical variations across altitude can not only give specific botanical identity to a species but can reveal interesting features helpful in understanding the range of morphological and anatomical variations present across different ecological zones. Annotations on different phenophases of the plant species in existing natural habitats along an altitudinal gradient helps to assess the role of environmental factors in *ex-situ* conservation and domestication[30] as the altitudinal gradients are considered among the most powerful natural experiments for testing ecological responses of biota to geophysical influence [31].

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