Journal of
Water & Climate Change

 Journal of Water and Climate Change Vol 15 No 2, 669 doi: 10.2166/wcc.2023.539

Accelerated anti-oxidant enzymes and phytochemical potential of *Taxus wallichiana* (Himalayan yew) under moist temperate forest of Himalayan, Pakistan

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ABSTRACT

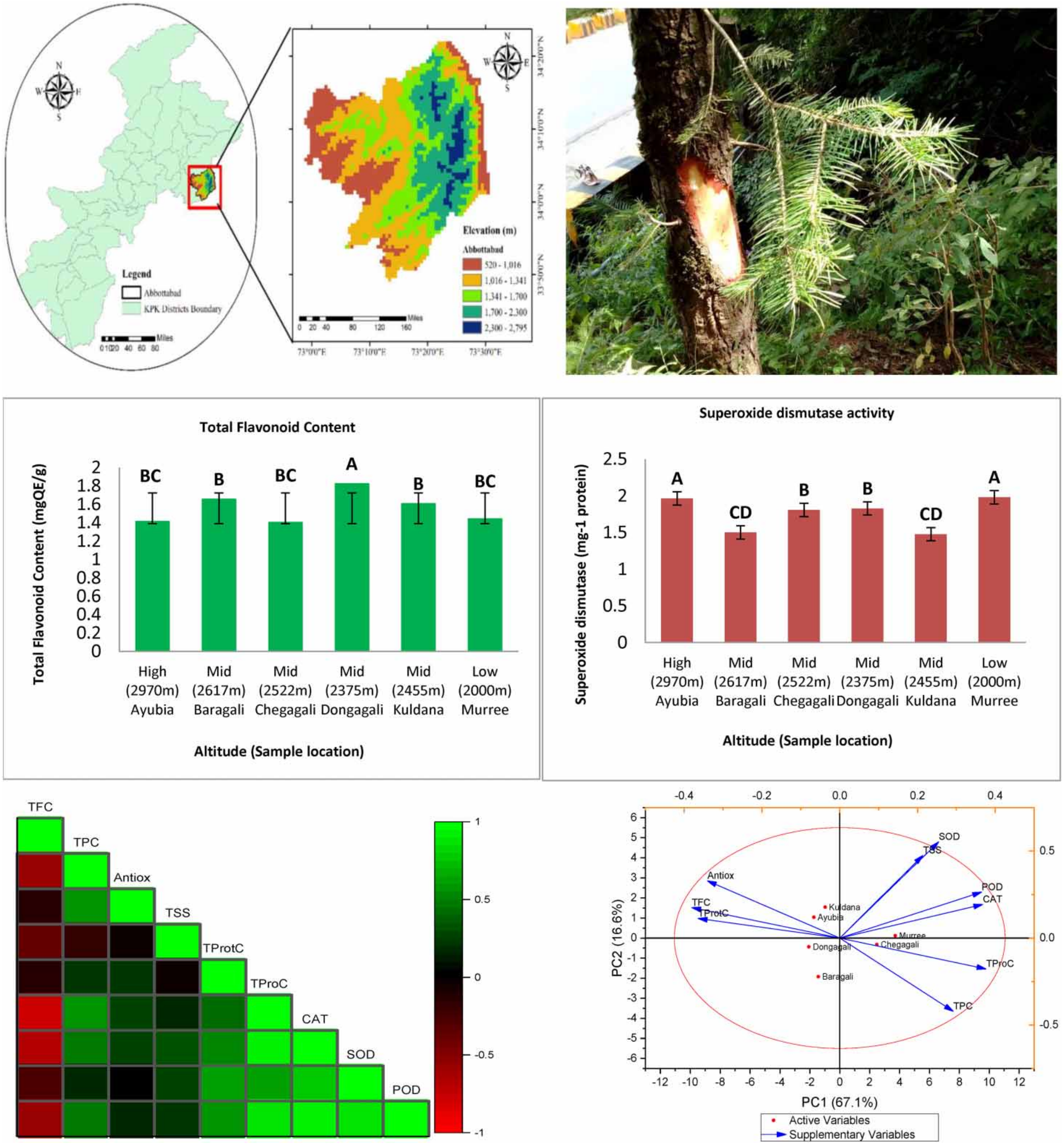
Taxus wallichiana (Himalayan yew) antioxidant potential enhances the release of secondary metabolites and enzymes under stress; over the last few decades owing to changes in climatic regimes, such species are under constant threat in the moist temperate Himalayan forests. The present study aims to evaluate the effect of change in land-use pattern on the antioxidant and phytochemical potential of *T. wallichiana* (Himalayan yew) in the moist temperate Himalayan Forest of Galiyat-Khyber-Pakhtunkhwa-Pakistan. Three leaf samples from each location of *T.W* were collected from high (Ayubiya, 2,970 m.a.s.l.) undisturbed, disturbed mid (Baragali, Dongagali, Kuldana, Chegagali, 2,617, 2,375, 2,455, 2,804 m.a.s.l.) and low (Murree, 2,000 m.a.s.l.) altitudes of moist temperate forest of Galiayt-Himalayan-Khyber-Pakhtunkhwa-Pakistan, DPPH assay, total flavonoids and phenolic content, total protein and proline content, catalase, superoxide dismutase and peroxidase activities were analysed. The antioxidant activity (DPPH) response was more pronounced in low and mid altitude disturbed sites than the undisturbed site at high altitudes. Antioxidant enzymes and osmolyte content further supported the stress tolerance capacity of *T. wallichiana* to scavenge the ROS produced under oxidative stress conditions. In conclusion, *Taxus wallichiana* inhabiting in these sites could withstand long durations of drought, salinity, frost, high temperatures, and pathogenic attacks by activating the antioxidant enzymes.

Key words: antioxidant enzymes, physio-biochemical, reactive oxygen species, *Taxus wallichiana* (Himalayan yew)

HIGHLIGHTS

- The biggest threat to the *Taxus wallichiana* population is overexploitation, deforestation and changing climatic regimes leading to droughts.
- *Taxus wallichiana* leaf samples collected from Ayubia and Murree regions showed maximum levels of antioxidant enzymes and osmolyte content thus validating the scientific fact that *Taxus wallichiana* growing in these regions are stress-tolerant and can withstand long duration of oxidative stress.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Moist temperate forests of Himalayan are enriched with diverse species and communities some of which have pharmacological, antioxidant and ethnobotanical potential (Adhikari & Pandey 2017; Liu *et al.* 2019; Rathore *et al.* 2019; Takshak & Agrawal 2019; Choudhari *et al.* 2020). *Taxus wallichiana* (Himalayan yew) is an important medicinal plant, which produces several secondary bioactive metabolites groups mainly amides, alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids and phenolic compounds and enzymes in medicinal plants (Larayetan *et al.* 2019; Mishra *et al.* 2022). Numerous environmental cues that plants encounter result in significant reductions in crop productivity. Variations in environmental conditions make it difficult for plants to reach their full genetic potential in terms of growth and reproduction. In addition to abiotic stress, such as drought, salinity and heavy metal stress, biotic stress in the form of microbial pathogens and herbivores on plants is one example of such an environmental state. Plants have evolved a wide range of intricate defense mechanisms in the form of accumulating secondary metabolites and activating antioxidant defense systems to fend off such attacks (Haque *et al.* 2022). These metabolites have antioxidant and pharmacological activities that protect plants from oxidative stress, and pathogenic and fungal attack (John *et al.* 2014; Zargoosh *et al.* 2019; Hashim *et al.* 2020). Their anticancer activities inhibit enzymes which activate carcinogens in human beings (Dhankhar *et al.* 2020; Iqbal *et al.* 2020). Antioxidant and phytochemical potential of plants is influenced by changes in climatic variables (Yang *et al.* 2018). Moist temperate forests of Himalayan in Galiyat are under constant threat owing to extensive deforestation and changes in land-use patterns thus causing ecological disturbance (Singh & Sharma 2020). Numerous plant species are eradicated and few are reported as endangered species in which *T. wallichiana* (Himalayan yew) is also included (Abbas *et al.* 2013; IUCN 2017). Thus, under such stress conditions, *T. wallichiana* (Himalayan yew) growth and production of secondary metabolites are greatly affected (Yang *et al.* 2016). Under oxidative stress conditions, plants tend to produce free radicals mainly including reactive oxygen species (ROS), as a result, the release of flavonoids and phenolic compounds that protect plants from oxidative stress is affected (Naz *et al.* 2020). Pakistan is located in an arid or semiarid region of the world, which puts it at risk for water stress issues just like any other developing country in this region. Drought is a concern on 1/4 of Pakistan's territory or 4.9 million hectares, and it is becoming worse every day (Qamar *et al.* 2018). Plants use a range of stress-coping strategies to deal with oxidative stress, including modifying the architecture of their shoots and roots, producing stress hormones like ethylene and abscisic acid, and activating antioxidant enzymatic defense systems (Iqbal *et al.* 2023). The main issue the plant defense system addresses is ROS brought on by habitats under stress regimes. To provide abiotic stress tolerance, an antioxidant system needs to be quick, strong and efficient. Hormones, various biochemicals and enzymatic activity all help to lessen and repair ROS damage. Enhancing the antioxidant system allows ROS absorption to reduce electrolyte leakage and lipid peroxidation, preserving the integrity and viability of cellular organelles and membranes (Haq *et al.* 2023). Endogenous superoxide dismutase (SOD) is synthesized in mitochondria in response to oxidative stress, with the primary purpose of neutralizing free radicals (Ismy *et al.* 2022). Changes in land-use patterns disrupt the ecosystem and raise air temperatures, which cause the glaciers to melt in the summer and increase precipitation and air temperatures, which in turn impact the soil microclimate (Mironova *et al.* 2022), nutrient content and microbial activity necessary for enhanced antioxidant potential of plants (Potashkina & Koshelev 2022).

Numerous studies in past were focused on estimating the antioxidant, antimicrobial and anticancer activities of *T. wallichiana* (Himalayan yew) and other medicinal plants growing in the moist temperate forests of Himalayan-Pakistan (Mehmood *et al.* 2021; Nazakat *et al.* 2021; Yousaf *et al.* 2022). Similarly, altitude was considered as an important parameter to evaluate species diversity and distribution in the moist temperate forests of Himalayan-Pakistan (Amin *et al.* 2023). Nonetheless, information about establishing a correlation between antioxidant activities and climatic variables with soil microclimate has not been reported yet in *T. wallichiana* (Himalayan yew) growing in the moist temperate Himalayan Forest of Galiyat-Pakistan.

The purpose of this study was to examine the relationship between changes in physio-biochemical attributes of *T. wallichiana* at different elevations under changing meteorological factors and soil microclimatic variables in the moist temperate forests of Galiyat (Abbotabad and Murree), Pakistan. In addition, determining the antioxidant activity (DPPH), antioxidant enzymes (CAT, SOD, POD) and metabolites (total phenol, flavonoids, soluble protein and proline contents) in the leaves of *T. wallichiana* (Himalayan yew) at different altitudes as well as their release under stress at high, mid and low altitudes were the main objectives of the present research study. The novelty of this study was established from the percentage of DPPH, total phenol and flavonoid content from the leaf extracts of *T. wallichiana* (Himalayan yew) growing in the disturbed forests. A low percentage under warming and disturbance may provide a deep insight into their slow vegetation growth and regeneration potential.

2. MATERIAL AND METHODS

2.1. Physiography of the area of study

Galiyat is geographically situated between latitude and longitude of 34.0642° N, 73.4062° E and 33°54′30.10″ N 73°23′25.08″ E within an altitudinal gradient of 1,500–3,400 m (Figure 1). The average annual temperature is 11.2 °C within an average rainfall of 1,448 mm annually. Galiyat is a hub of diverse shrubs, herbs and medicinal plants with pharmacological and ethnobotanical

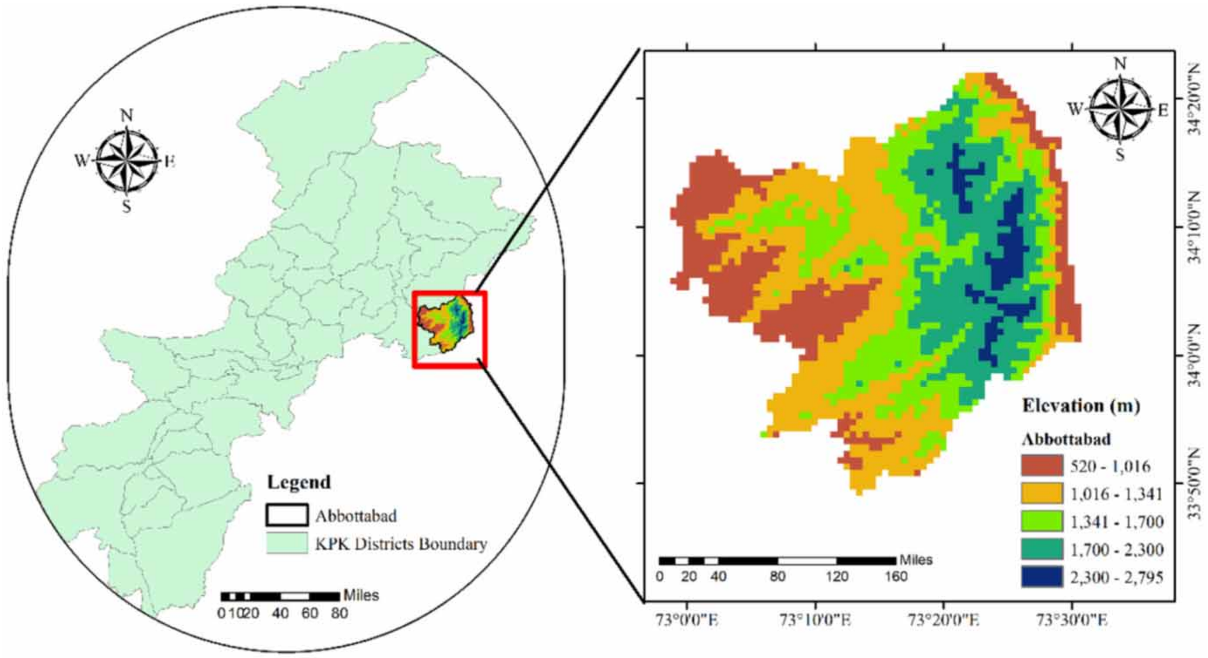


Figure 1 | Showing the area of study from which the samples were collected (Goheer *et al.* 2022).

potential. The Murree region possesses a temperate climate with cool summers and cold winters. June is the hottest month, while January and February are the coldest months. For June, the mean maximum and minimum temperatures are 26.6 and 13.3 °C, respectively, soils are predominantly sandy clay, with good moisture content, and rich in organic matter (Siddiqui 2011).

2.2. Leaf and soil samples collections and analysis

T. wallichiana (Himalayan yew) leaves samples were collected from high altitude (Ayubia, 2,970 metres above sea level (m.a.s.l.)), mid altitudes (Bara Gali, 2,617 m.a.s.l., Dungagali 2,375 m.a.s.l., Kuldana, 2,455 m.a.s.l., Chengagali, 2,522 m.a.s.l.) and low altitude (Murree, 2,000 m.a.s.l.) of moist temperate Himalayan forests of Galiyat-KP-Pakistan (Figure 1).

Each leaf sample was air-dried and then washed thoroughly with sterile water for further analysis by adopting the method of Bogers *et al.* (2006).

2.3. Estimation of total flavonoid and phenolic content

Total flavonoid content (TFC) was determined using a plant flavonoids colorimetric assay kit and the detection principle is based on the reaction between the flavonoids and the aluminum ion resulting in a red complex (Siddiqui *et al.* 2017). Total phenolic content (TPC) was determined by the Folin–Ciocalteu method (Stefănescu *et al.* 2020). For quantitative determination, a gallic acid calibration curve was prepared with solutions ranging in concentration from 50 to 450 μg/ml ($R2 = 0.9994$). Absorbance was measured at 765 nm (Siddiqui *et al.* 2017). Results were expressed as mg GAE (gallic acid equivalent) per 100 g of FWF. Assays were performed in triplicate using a SPECTROstar® Nano microplate spectrophotometer (BMG Labtech, Ortenberg, Baden-Württemberg, Germany).

2.4. Antioxidant activity

The free radical neutralizing activity of samples and standard solutions of ascorbic acid in methanol was determined by their ability to react with the stable free radical 1,1-diphenyl-2-picryl group (DPPH) (Blois 1958). Plant samples at various concentrations (15–250 μg/ml) were added to a 100 μM solution of DPPH in ethanol. After incubation at 37 °C for 30 min, the absorbance of each solution was measured at 517 nm. The measurement was performed three times. Free radical scavenging activity was calculated using the equation: $\%\ of\ free\ radical\ scavenging\ activity = (A_0 - A_T) \times 100\ A_0$, where A_0 is the absorbance of DPPH solution and A_T is the absorbance of test or reference sample.

2.5. Soluble sugar content

A homogenized mixture was prepared by taking fresh leaf material (0.5 g) and grinding in 5 ml of distilled water. Samples containing the homogenized mixture were placed in a centrifuge and centrifuged for 10 min. After the centrifugation process, 1 ml of the supernatant was taken from each sample and 4 ml of concentrated (35%) H_2SO_4 was added. The optical density (OD) was determined at 490 nm according to the procedure (Dubois *et al.* 1956).

2.6. Total protein content

The protein content of leaf tissue was studied according to the protocol of Bates *et al.* (1973). 0.5 g of fresh leaf tissue was ground with the help of ice cold mortar and pestle in 5 ml phosphate buffer (pH 7.0). A homogenized mixture was obtained after grinding, and a sample of the prepared mixture was placed in a centrifuge and centrifuged was spun for 15 min. After centrifugation, 0.1 ml of the supernatant was taken from each sample and 2 ml of Bradford's reagent was added. OD was measured at 595 nm.

2.7. Total proline content

The content of proline was quantified according to the method of Bates *et al.* (1973). Fresh leaves (0.5 g) were ground in 10 ml of 3% aqueous solution of sulfosalicylic acid to obtain a homogenized mixture. The mixture was filtered and 2 ml of the filtrate was collected. Likewise, 4 ml of ninhydrin solution and 4 ml of glacial acetic acid (20%) were mixed with 2 ml of the filtrate taken. The mixture was heated at 100 °C for 1 h, to which 4 ml of toluene was added. OD readings were recorded at 520 nm.

2.8. Determination of CAT activity

Catalase activity (CAT) was determined according to the procedure of Tyburski *et al.* (2009). The reaction mixture (1 ml) contained 0.4 ml of 100 mM potassium phosphate buffer (pH 7.0), 0.4 ml of 30% H_2O_2 and 0.4 ml of enzyme extract. Decomposition of H_2O_2 within 3 min was measured as a decrease in OD at 240 nm.

2.9. Determination of SOD activity

The SOD content of fresh leaf materials was measured according to the standard procedure of Yuan *et al.* (2011). The reaction mixture (3 ml) contained 0.1 ml supernatant, 0.72 ml methionine (58 mg methionine in 30 ml distilled water), 0.72 ml HBT (1.89 mg NBT in 30 ml distilled water), 0.72 ml EDTA (1.1 mg EDTA in 30 ml distilled water) and 0.72 ml riboflavin (0.02 mg riboflavin in 30 ml distilled water). Samples were incubated for 30 min in the dark and then stored in light. OD readings were observed at 560 nm using a spectrophotometer.

2.10. Determination of POD activity

To estimate POD activity the method of Asthir *et al.* (2009) was followed. Fresh leaves (0.5 g) were mixed with 2 ml of the solution (12.5 g of PVP, 0.2 ml of phosphate buffer (pH 7.0) and 4.6 g of EDTA in 125 ml of distilled water) and centrifuged for 20 min. The reaction mixture (3 ml) contained 0.1 ml supernatant, 1.3 ml MES buffer (970 mg MES in 50 ml distilled water), 0.0.1 ml phenyldiamine (36 mg in 4 ml distilled water) and a drop of 0.2% H_2O_2. Absorbance changes were recorded at 485 nm for 3 min using a spectrophotometer.

2.11. Statistical analysis

SPSS Statistic-25 software was employed for the analysis of variance (ANOVA). By using standard techniques, mean and standard errors were calculated and the least significance difference (LSD) test at ($P \leq 0.05$) was performed and indicated by letters (a–g). Correlation analysis and principal component analysis (PCA) were performed by using R studio. The graphs were drawn by using SigmaPlot 14.5 software.

3. RESULTS

3.1. Estimation of total flavonoid and phenolic content

For the estimation of total flavonoid and phenolic contents (TFC and TPC), *T. wallichiana* (Himalayan yew) leaf samples were collected from high altitude (Ayubia, 2,970 m.a.s.l.), mid altitudes (Bara Gali, 2,617 m.a.s.l., Dungagali 2,375 m.a.s.l., Kuldana, 2,455 m.a.s.l., Chengagali, 2,522 m.a.s.l.) and low altitude (Murree, 2,291 m.a.s.l.) of moist temperate Himalayan forests of Galiyat-KP-Pakistan . TFC was 0.6 mg Qe per g at high and low altitudes, whereas at mid altitude, it ranged from 0.6 to

1.8 mg Qe per g and was significantly ($P < 0.05$) greater than low and high altitudes. Whereas TPC was significantly ($P <$ 0.05) greater at high altitude (2.7 mg GAE per g) and low (2.6 mg GAE per g) at mid altitude (2.2–1.4 mg GAR per g) except for TPC at one of the mid altitude sites (Kuldana) which were found to be same as at low altitude (Murree). There was no significant difference in TFC between high and low altitudes.

3.2. Antioxidant potential (DPPH%)

In comparison with the control group (ascorbic acid) which showed 97% inhibition; significant ($P < 0.05$) antioxidant activity was observed in plant leaf samples collected from low (Murree), high (Ayubia) and mid (Kuldana) altitudes, that counted for 93, 96 and 91% inhibition, respectively. However, it was observed that the DPPH% was significantly lower (20%) in the leaf extract of TW growing in the Chegagali of mid altitude with moderate soil moisture conditions than other TW of Baragali and Dungagali of mid altitudes. The % of DPPH was reduced to 70% from 80 to 90% at moderate soil moisture conditions as observed between the difference in the DPPH% between mid altitudes of four sites (Figure 2). Furthermore, DPPH% was more or less significantly the same in the leaf of TW growing at Baragali and Dungali of moderate soil moisture conditions at mid altitudes when compared with the TW leaf extract DPPH% of Ayubia with high soil moisture conditions at high altitude and low air temperature (Figure 2; Table 1).

3.3. Total soluble sugar, total protein and TProC

The total soluble sugar content (TSS), total protein (TProt) and total proline (TProl) content of *T. wallichiana* leaf were quantified. It was observed that TSS was 2.7 and 2.2 μmol/g at high and low altitudes, respectively, and ranged from 2.2 to 2.7 μmol/g at mid altitudes. TSS was significantly ($P < 0.05$) greater at high altitudes (2.7 μmol/g) than mid and low (2.45 and 2.2 μmol/g) altitudes (Figure 3). This was further supported by a positive (0.50) correlation with altitude (Figure 6; Table 1). Whereas total protein content (TProtC) and total proline content (TProlC) (0.6 and 2.6 μmol/g) were the same at high and low altitudes but were significantly ($P < 0.05$) greater than mid altitudes (0.4 and 2.1 μmol/g). TProtC was significantly high at moist and dry soil moisture conditions in the leaf extracts of TW growing at high and low altitudes of Ayubia and Murree study sites. Whereas TProtC of TW leaf extract growing in the four mid altitudes of Baragali, Chengagali, Dungagali and Kuldana was significantly lower ($P < 0.05$) than other high and low altitudes sites of Ayubia and Murree. However, TProtC was more or less the same among mid altitude sites with moist to dry soil conditions. Total protein content extracted from the leaf extracts of TW growing at Ayubia and Murree with moist and dry moisture conditions of high and low altitudes and of Chengagali and Kuldana with moderate soil moisture conditions was significantly ($P < 0.05$) was the same and greater than the TProlC of the leaf of TW growing at moderate soil moisture conditions of mid altitudes of Baragali and Dungagali.

3.4. Antioxidant enzymes (CAT, SOD and POD)

The activities of antioxidant enzymes including catalase (CAT), SOD and peroxidase were assessed. Significant ($P \leq 0.05$) activities of catalase and superoxide dismutase were observed in the leaf samples taken from high (Ayubia) and low (Murree) altitudes of the six sample location sites. In addition, significant ($P \leq 0.05$) peroxidase (POD) activity was observed in the *T. wallichiana* leaf samples collected from high (Ayubia), mid (Chegagali) and low (Murree) altitudinal gradients of the study site. However, it was observed that there was a significant difference ($P \leq 0.05$) in POD and SOD content between high and mid altitude except for Chegagali site of mid altitude where the POD content remained the same between high and low altitudes (Figure 4).

3.5. Correlation between phytochemical and physio-biochemical traits of *T. wallichiana* (Himalayan yew)

A Pearson's correlation histogram was constructed to show a correlation between the phytochemical and physio-biochemical traits of *T. wallichiana.* A positively significant ($P \leq 0.05$) correlation was noted among physio-biochemical and phytochemical attributes of *T. wallichiana* including total proline content (TProC), TProtC and the activities of antioxidant enzymes including peroxidase (POD), SOD and catalase (CAT); Similarly, TSS correlated positively with TProC and all the antioxidant enzymes. In addition, antioxidant activity and TPC showed a positive correlation with TProC, TProtC, peroxidase (POD) and catalase (CAT) activity. However, TFC showed a significantly ($P \leq 0.05$) negative correlation with TSS, TPC, TProC and all the antioxidant enzymes (Figure 5).

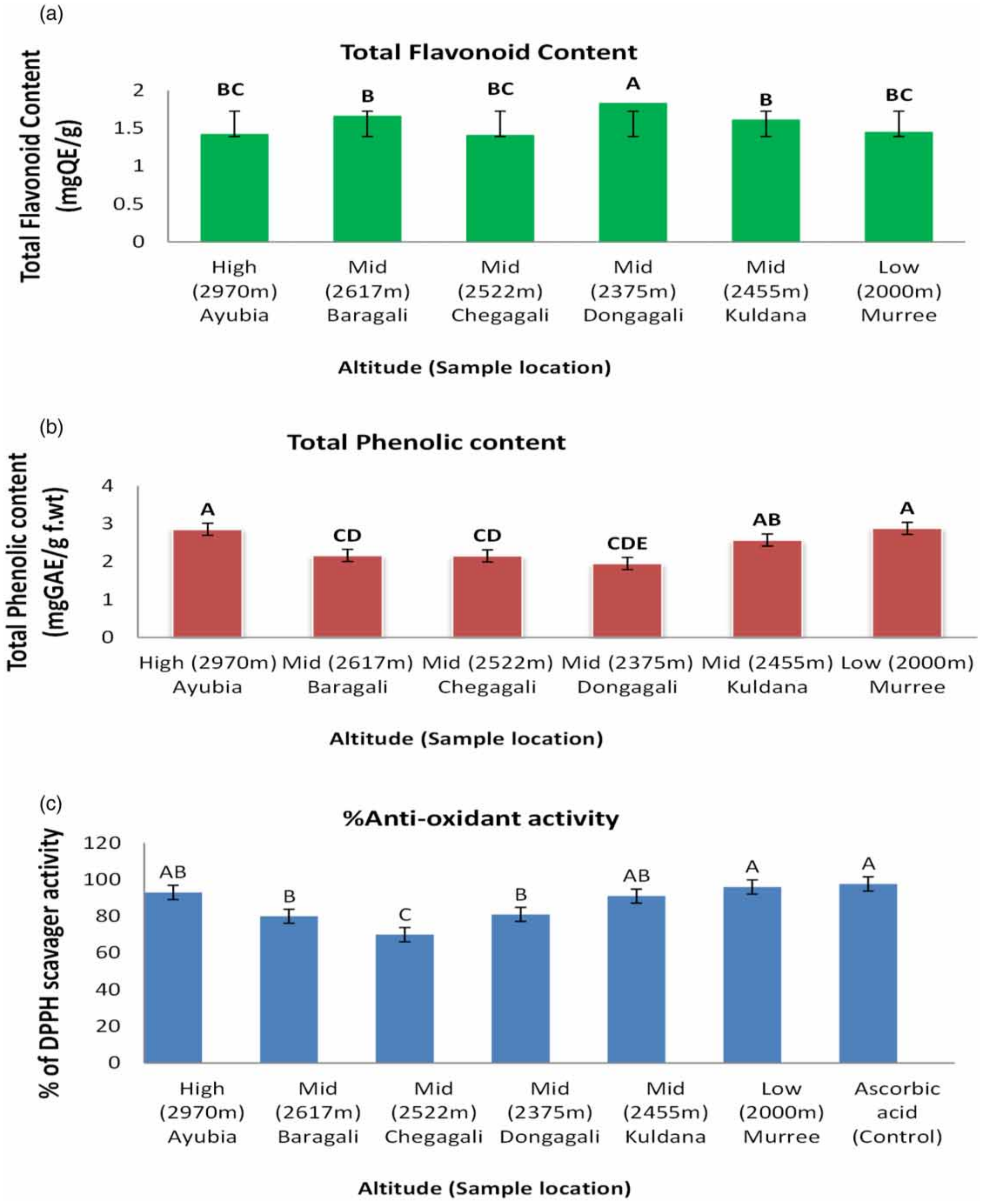


Figure 2 | Showing (a) total flavonoid content (TFC), (b) total phenolic content (TPC), (c) % antioxidant activity of leaf samples collected from different localities of the area of study (mean $\pm$ standard error). Letters (A–E) indicating least significance difference among the mean values at $p \geq 0.05$.

3.6. Principal component analysis

The loading plots of PCA show % variations in the total data. In the whole database, PC1 and PC2 contributed the maximum portion and occupied 83.7% of all databases. Among these PC1 exhibited (67.1%) and PC2 exhibited (16.7%). All studied attributes were distributed successfully in the database which clearly depicts a positive correlation among maximum physio-biochemical and phytochemical attributes of *T. wallichiana* including TProC, TProtC and the activities of antioxidant enzymes such as POD, SOD and CAT. Similarly, TSS correlated positively with TProC and all the antioxidant enzymes in the whole database. In addition, antioxidant activity and TPC showed a positive correlation with TProC, TProtC, peroxidase (POD) and catalase (CAT) activity in the overall database. However, TFC was negatively correlated in the database to TSS, TPC, total proline content (TProC) and all the antioxidant enzymes (Figure 6).

Table 1 | Showing altitudes of different sites of the area of study, soil moisture regime and physio-biochemical and phytochemical attributes of *Taxus wallichiana* leaf

Sites	Altitude (m)	Soil moisture regime	Total flavonoid content (Mg Qe/g)	Total phenolic content (mg GAE/g)	% Antioxidant activity	Total soluble sugar (μg mol g^{-1})	Total protein content (μg mol g^{-1})	Total proline content (μg mol g^{-1})	CAT (mg^{-1} protein)	SOD (mg^{-1} protein)	POD (mg^{-1} protein)
Ayubia	High (2,970)	Udic	1.4	2.8	93	2.7	1.9	2.6	2.8	1.9	1.9
Baragali	Mid (2,617–2,375)	Xeric	1.6	2.1	80	2.2	1.4	1.6	2.0	1.5	1.2
Chegagali			1.4	2.1	81	2.4	1.6	2.3	2.3	1.8	1.8
Dongagali			1.8	1.9	91	2.7	1.4	1.7	2.0	1.8	1.1
Kuldana			1.6	2.5	70	2.7	1.2	2.3	2.3	1.4	1.6
Murree	Low (2,000)	Xeric	1.4	2.8	96	2.2	1.9	2.6	2.8	1.9	1.9

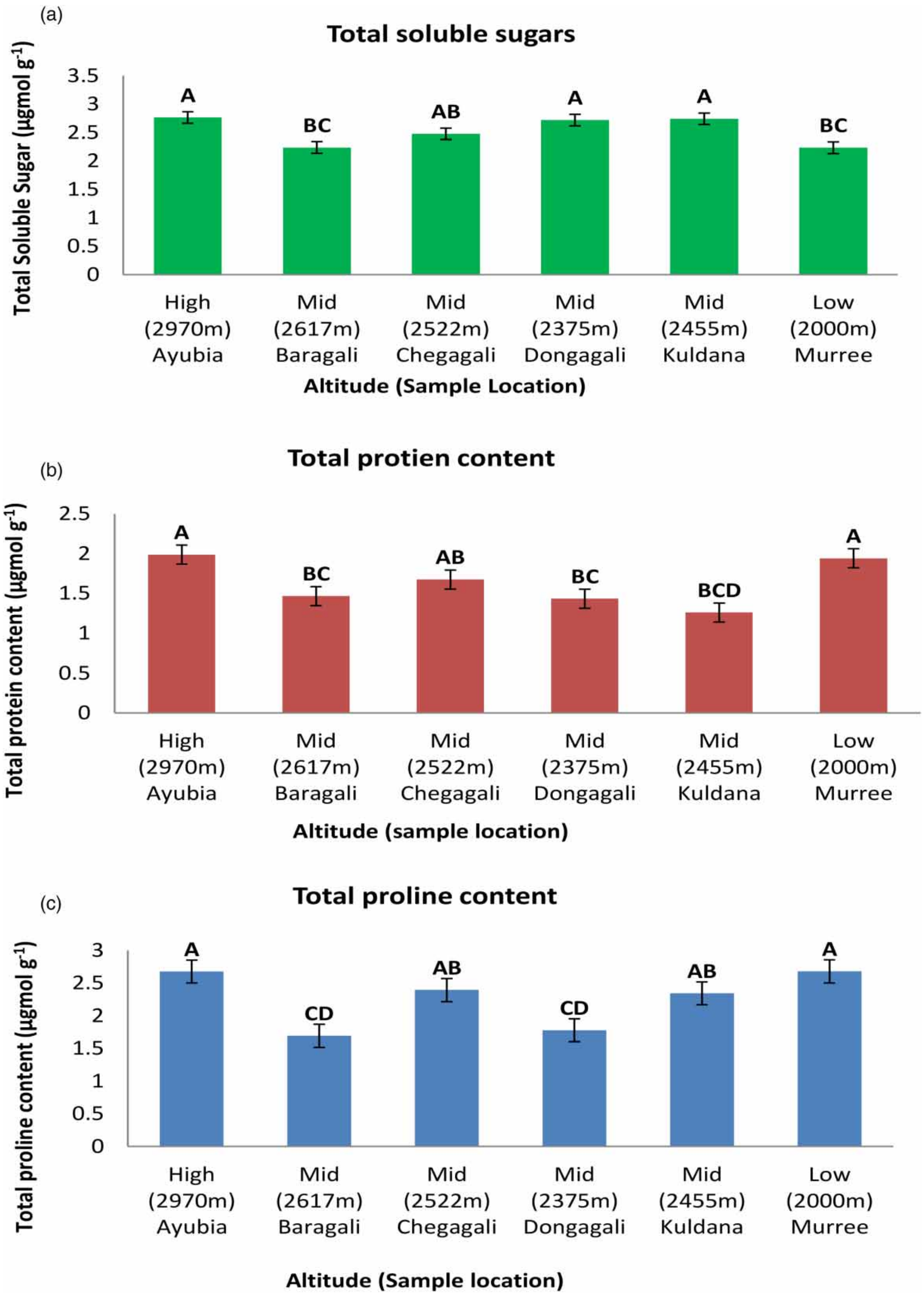


Figure 3 | Showing (a) total soluble sugar (TSS), (b) total protein content (TProtC), (c) total proline content (TProC) of leaf samples collected from different localities of the area of study (mean ± standard error). Letters (A–D) indicating least significance difference among the mean values at $p \geq 0.05$.

4. DISCUSSION

T. wallichiana (Himalayan yew) is an endangered species and eradicated rapidly from moist temperate Himalayan forests of Galiyat-KP-Pakistan (IUCN 2017). This species because of its antioxidant potential that enhances its defense mechanisms

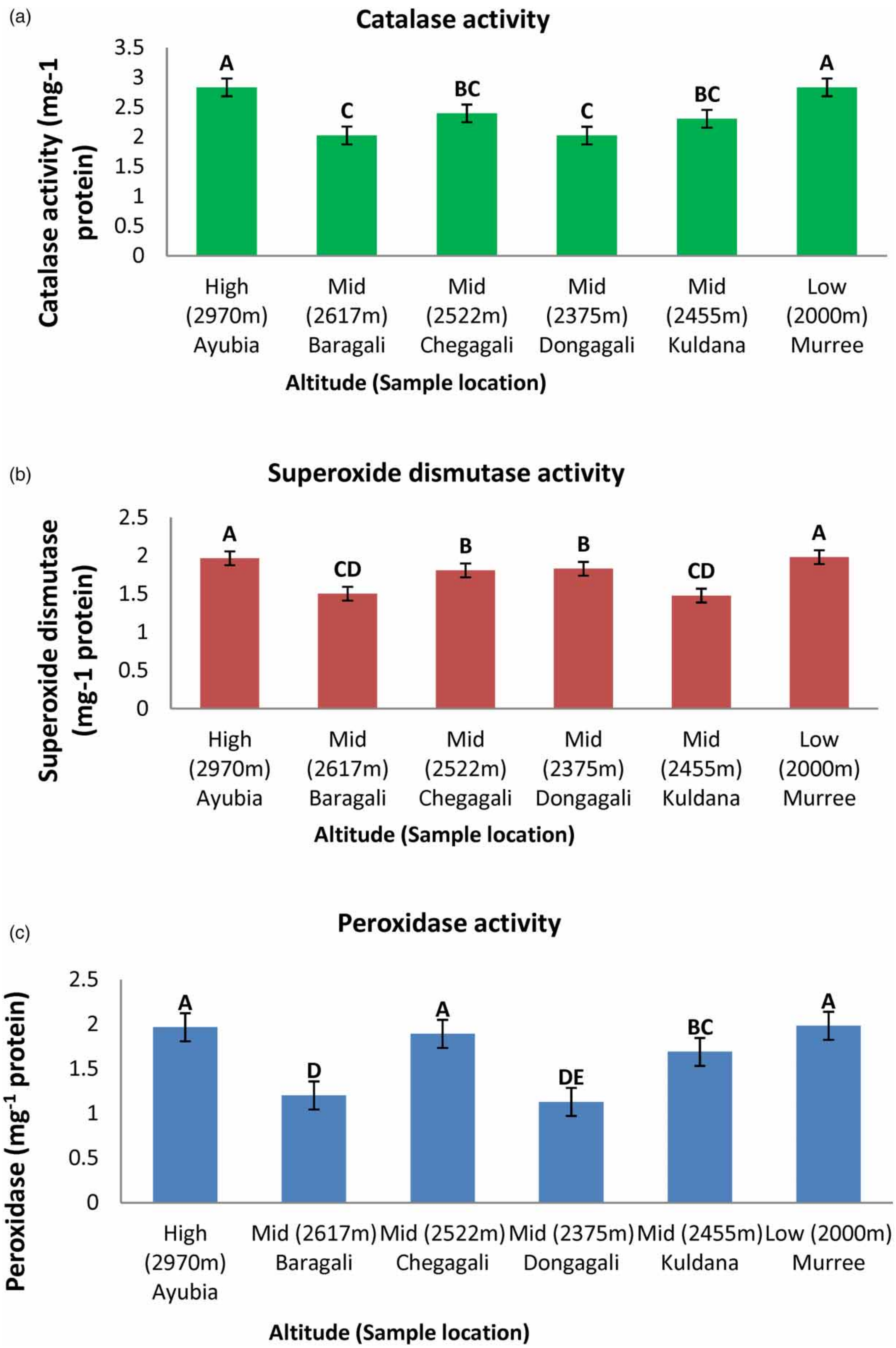


Figure 4 | Showing (a) catalase activity (CAT), (b) superoxide dismutase activity (SOD), (c) peroxidase activity (POD) of leaf samples collected from different localities of the area of study (mean ± standard error). Letters (A–E) indicating least significance difference among the mean values at $p \geq 0.05$.

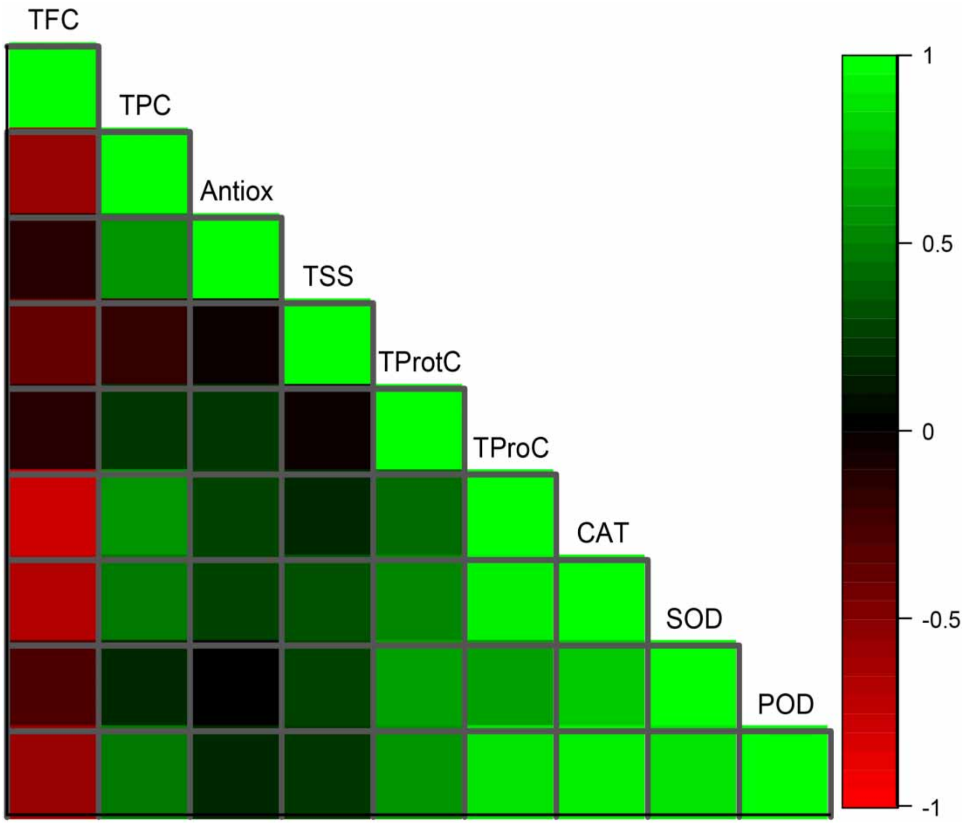


Figure 5 | Correlation between phytochemical and physio-biochemical traits of *Taxus wallichiana.* Total flavonoid content (TFC), total phenolic content (TPC), antioxidant activity (Antiox), total soluble sugar (TSS), total protein content (TProtC), total proline content (TProC), catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD).

against oxidative stress and also protects it from the harmful effect of these free radical production or scavenging free radicals is categorized as exotic species (Bhat *et al.* 2018). It is well understood that Himalayan yew grows at an altitudinal gradient of 3,400–1,800 m.a.s.l. in moist temperate Himalayan forests of Galiyat-KP-Pakistan. Altitude is considered an important factor that may affect the antioxidant, and phytochemical potential of Himalayan yew (Jugran *et al.* 2016; Adhikari & Pandey 2020). This is more likely because the altitude affects the climate and soil microclimate which indirectly affects the antioxidant and phytochemical potential of *T. wallichiana* (Himalaya yew) growing at altitude. At high altitudes, the air temperature is much cooler and soil moisture content is higher than at low altitudes. Similarly high altitude is undisturbed than low and mid altitude where *T. wallichiana* (Himalayan yew) was growing at the side of the road. Furthermore, soil moisture and organic matter content are also affected by altitude. The results of this study show that soil moisture content and organic matter were positively correlated with altitude and also with antioxidant scavenging activity against free radicals. This suggests that moisture content is the most important factor that affects the antioxidant potential of *T. wallichiana* (Himalayan yew). Phenolic and flavonoid contents are secondary metabolites and sequestered free radicals and play an important role in the chelation of elements (Becker *et al.* 2019), thus they are very important in enhancing the antioxidant activity of the species. The antioxidant activity of the leaf extract of *T. wallichiana* was probably due to the presence of bioactive phytochemicals such as polyphenols, flavonoids, terpenoids and saponins, our findings were parallel with the results of Guleria *et al.* (2013) who observed a prominent antioxidant activity of *T. wallichiana* methanolic leaf extracts by significantly inhibiting pathogenic bacterial strains. In addition, our results were in agreement with those of Milutinović *et al.* (2015) which mentioned that the *Taxus* methanolic extracts have potent antioxidant properties and act as free radical scavengers. It was observed in this study that TFC was positively correlated with moisture but not with organic matter content. Thus, moisture content is the most important factor that affects the antioxidant potential and TFC in TW. Total soluble solids are also an important factor which is positively correlated with altitude, soil moisture and organic matter contents.

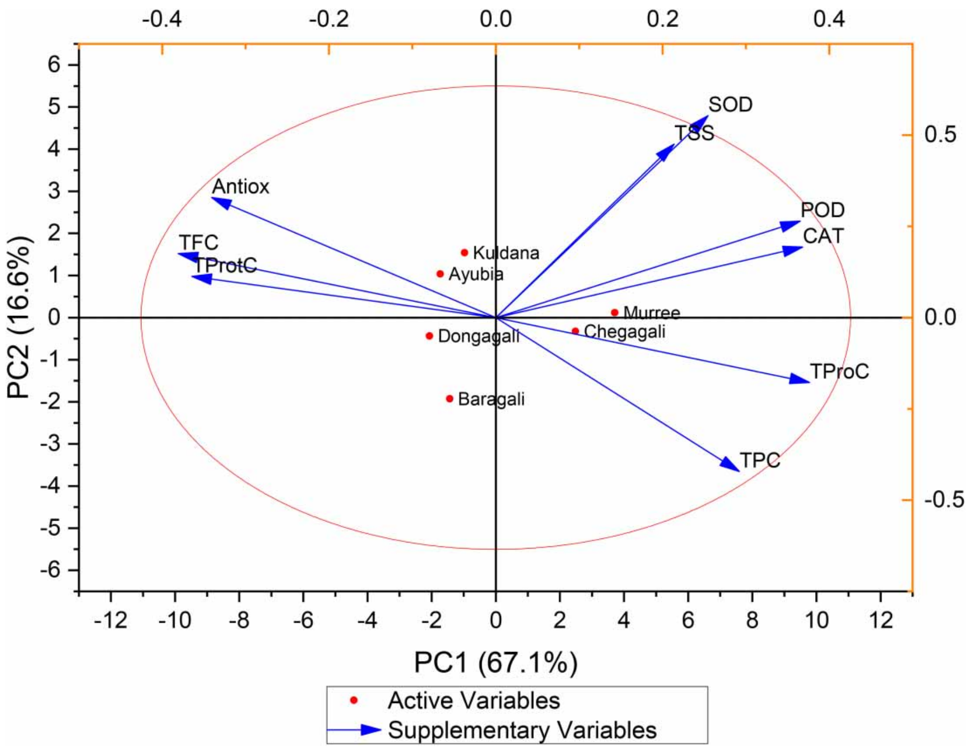


Figure 6 | Loading plot of principal component analysis (PCA) of various phytochemical and physio-biochemical attributes of *Taxus wallichiana.* Total flavonoid content (TFC), total phenolic content (TPC), antioxidant activity (Antiox), total soluble sugar (TSS), total protein content (TProtC), total proline content (TProC), catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD).

This may enhance the ability of adsorbed elements to be released in the soil solution and thus become available to the plants for their growth and development. The greater content of TSS in soil revealed the contention that such soils have more soluble ions in their solution than dry soil. It was noted in this study that TSS was highly positively correlated (0.72, 0.70) with soil moisture and organic matter content thus enhancing the antioxidant potential of the TW. Whereas all enzymes CAT, SOD and POD were highly correlated with each other whereas antioxidant activity was found to be positively correlated with TProlC and CAT, TProtC was positively and highly correlated with TProlC, CAT, SOD and POD. whereas TSS increases the solubility of the elements in soil solution thus enhancing their transport in plants. Air temperature is cool at high altitudes than at low altitudes; therefore, altitude is also an important factor that may affect the *T. wallichiana* (Himalayan yew) antioxidant activities which are enhanced by antioxidant enzymes (SOD, POD and CAT) and metabolites like phenolic and flavonoids compounds and glutathione which chelate iron (Fe) ions (Bhat *et al.* 2018; Dumitraş *et al.* 2022).

Phenolics are a range of different secondary metabolites and have the great ability to sequester free radicals or chelation of metal ions. It is observed that antioxidant activity is directly correlated with TPC in the plants. This study also shows that antioxidant activity was highly positively correlated (0.79) with phenolic content. This is supported by previous studies performed by Qader *et al.* (2011), Rawat *et al.* (2011), Saeed *et al.* (2012), and Jugran *et al.* (2016), who studied the correlation between antioxidants and phenolic compounds in diverse medicinal plants of Himalayan Forest. It was observed in this study that altitude had no effect on any of the antioxidants or phytochemical activities except for TSS. TSS was found to be positively correlated with altitude whereas TFC, antioxidants, CAT, SOD and POD was weakly negatively correlated with altitude. Total phenol content (TPC), TProtC and TProlC was weakly positively correlated with altitude (2,794–2,000 m.a.s.l.). Adhikari & Pandey (2020) studied the correlation between air temperature, soil moisture, rainfall, altitude (1,975, 2,434 and 3,106 m.a.s.l.) and nitrogen, phosphorus and carbon on antioxidant activities by measuring DPPH inhibition, total phenols, flavanols, flavonoids and tannins in the needles of *T. wallichiana* Zucc (Himalayan yew) of temperate forests of Kashmir Himalayan-India. The total phenol, flavonoid content and bioactive compounds such as gallic acid, ascorbic acid and quercetin and DPPH were significantly ($P < 0.01$) positively correlated with altitude ($P < 0.01$), rainfall and moisture but negatively correlated with temperature. This is a very important observation which suggests that the greater percentage of DPPH in the leaf extract under both very moist and dry conditions may accelerate the oxidative stress potential of TW. This further suggests that leaf extract of TW may be used as a herbicide to protect TW from pathogenic attack and fungal attack. This may enhance the TW regeneration potential and vegetation growth under stress.

The results of this study are in agreement with the findings of Adhikari & Pandey (2020) and Kirakosyan *et al.* (2004) who reported that *T. wallichiana* phenolic content was positively correlated with soil moisture, soil organic carbon and altitude. They concluded that moisture accelerates the synthesis of polyphenols whereas altitude and organic carbon enhance the release of phenolic compounds from plants under water-sufficient conditions or at high moisture content. Antioxidant activity is directly linked with high phenolic content. Soluble sugars and proteins have been reported as effective osmo-protectants in the defense system against abiotic stress in plants (Mutava *et al.* 2015). At the onset of stress conditions, plants tend to accumulate various types of osmolytes such as sugars, proteins, proline and glycine betaine. Osmolytes chiefly accumulate in the cytoplasm preventing cellular degradation and maintain osmoregulation. Owing to their nontoxic nature and high solubility they do not impede other physio-biochemical processes (Wahab *et al.* 2022). Soluble carbohydrates and proteins assist in retaining water content in the cells and tissues and avoid desiccation under abiotic stress regimes. In such conditions, the breakdown of complex carbohydrates (polysaccharides) leads to the production of low molecular weight soluble sugars including glucose, galactose, fructose and sucrose. The research findings made by Dugasa *et al.* (2019) and Ullah *et al.* (2021) were in agreement with our result which concluded that flax (*Linum usitatissimum*) plants grown under salinity and drought stress caused a marked increase in soluble protein content. Likewise, Orabi *et al.* (2016) in faba bean (*Vicia faba*) also found a prominent increase in total protein concentration cultivated under abiotic stress conditions. Proline is well known for its osmotic protective role. In many plants, increased proline concentrations under drought stress conditions have been shown to be associated with drought stress tolerance (Khan *et al.* 2020). It is also clear that proline can act directly as a ROS scavenger and regulator of the redox state of cells (Shah *et al.* 2022). Proline synthesis helps reduce osmotic pressure in the cytoplasm, maintain the NADP/NADPH + ratio enhance cellular water uptake and protect cell growth (Javed & Ikram 2008; Kakar *et al.* 2023). In addition, proline acts as an osmolyte, free radical scavenger and ROS scavenger, macromolecular stabilizer and cell wall component. As a chemical protectant, it withholds the integrity of cell membranes by stabilizing the conformation of proteins, inhibiting the degeneration of natural enzymatic compounds and preventing lipid peroxidation (Hayat *et al.* 2012). Our results are consistent with a study on Chinese ryegrass (*Leymus chinensis*) that showed high levels of proline in drought-stressed seedlings. Identical results have been reported for soybean (*Glycine max*) and faba bean (*V. faba*) (Mustafa *et al.* 2015).

The extent of cell damage under stress depends on the rate of free radical and ROS production and the efficiency of plant detoxification mechanisms (Dugasa *et al.* 2019; Shah *et al.* 2021; Shumaila *et al.* 2023). To combat oxidative stress, plants have highly effective antioxidant defense systems that can destroy, neutralize or scavenge free radicals. These systems include antioxidant enzymes, such as CAT, SOD, APX, GPX, POD and GR, and non-enzymatic antioxidant systems such as ascorbate, alpha-tocopherol, carotenoids, phenolic compounds, proline and glutathione (Blokhina *et al.* 2003; Król *et al.* 2014). SOD is the first and most important enzyme in the detoxification of ROS compounds that protect cells from the risk of OH radical formation by converting O_2 radicals to H_2O_2 in the cytoplasm, chloroplasts and mitochondria (Alscher *et al.* 2002; Diaz-Vivancos *et al.* 2013). The H_2O_2 produced is then broken down by enzymes, such as CAT, GPX and APX in the next step (Hamanaka & Chandel 2009).

5. CONCLUSIONS

The present investigation concluded that the *T. wallichiana* (Himalayan yew) antioxidant potential was accelerated under oxidative stress and high soil low moisture content. Moreover, Antioxidant potential (DPPH) activates and secondary metabolites (phenolic compounds) were positively correlated with soil moisture and organic carbon contents which suggested that *T. wallichiana* (Himalayan yew) may tolerate highly moist soil conditions. The results further concluded that *T. wallichiana* (Himalayan yew) may tolerate oxidative stress and that it could withstand long durations of drought, salinity, frost, high temperatures and pathogenic attacks by activating the antioxidant enzymatic défense system and accumulating osmolytes. All the leaf samples collected from high, mid and low altitudes, high activities of antioxidant enzymes including CAT, SOD and POD and maximum accumulation of osmolytes (TProC and TProtC) were found in the leaf samples taken from low altitude

(Murree) and high altitude (Ayubia) regions, which validated the fact that *T. wallichiana* species inhabiting in these locations could be more tolerant to oxidative stress as compared to the rest of the sample sites having *T. wallichiana* species. Furthermore, the leaf extract with an accelerated antioxidant enzymatic défense system may improve the accumulation of osmolytes and protect the plants from abiotic stress.

ACKNOWLEDGEMENT

We the authors are grateful for the financial and moral support of the National Center for Excellence in Geology Peshawar and Higher Education Commission Pakistan for this Research work.

AUTHOR'S CONTRIBUTION

Sanam Zarif was the principal author who designed the research, performed the experiments and wrote the manuscript; Samina Siddiqui and Asim Shahzad were supervisor and co-supervisor who helped in the formal analysis and investigation and write up of manuscript, reviewed and helped in editing of the manuscript, while Wadood Shah helped in writing the manuscript, editing, revision and statistical analysis. All authors have read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 25 September 2023; accepted in revised form 18 December 2023. Available online 29 December 2023