Research Article
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Climate-Induced Variability in Secondary Metabolites of *Indigofers heterantha:* Implications for Conservation and Sustainable Mountain Agriculture

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Abstract

Mountain ecosystems are highly vulnerable to climate change, which threatens the survival, distribution, and medicinal properties of indigenous plant species. *Indigofera heterantha* (*I. heterantha*), an important Himalayan medicinal shrub, is traditionally used by mountain communities to treat various ailments. The present study was conducted to compare bioactive secondary metabolites and antioxidant activities between wild-grown and in vitro propagated plant materials of *I. heterantha* to explore sustainable conservation and climate-resilient utilization strategies for mountain agricultural systems. Young, healthy leaves, flowers, and roots of naturally growing plants, along with shoots and adventitious roots produced through in vitro culture techniques, were analyzed for total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical-scavenging activity (DRSA). Comparatively lower amounts of TPC and TFC were observed in the in vitro propagated plants. The highest total phenolic and flavonoid contents were recorded in root samples collected from the wild habitat, containing 3623.89 and 468.89 µg g^{-1} dry weight (DW), respectively. Among the in vitro propagated materials, adventitious roots showed relatively higher TPC (2873.89 µg g^{-1} DW) and TFC (360.81 µg g^{-1} DW), followed by in vitro-induced roots with TPC and TFC values of 1420.19 and 308.28 µg g^{-1} DW, respectively. Micro propagated shoots exhibited TPC and TFC values of 114.63 and 201.21 µg g^{-1} DW, respectively, compared with wild-collected leaves containing 65.56 and 191.11 µg g^{-1} DW. The highest DPPH radical-scavenging activity (63.43%) was recorded in leaves collected from the natural habitat, whereas in vitro-induced roots showed a DRSA of 46.75%. The lowest DPPH radical scavenging activity (30.63%) was observed in wild-collected root extracts. The findings indicate that environmental conditions and abiotic stresses associated with mountain ecosystems strongly influence secondary metabolite biosynthesis in *I. heterantha*. The study highlights the importance of integrating plant tissue culture and conservation biotechnology to support the sustainable use and climate-resilient conservation of medicinal plants in mountain agricultural systems.

Keywords: Climate change, Mountain ecosystems, Himalayan medicinal plants, *Indigofera heterantha*, Medicinal plant, Antioxidant Activity, Total Phenolics, Total Flavonoids,

Highlights

- Comparative evaluation of secondary metabolites and antioxidant activity between wild-grown and in vitro propagated *I. heterantha*.
- Wild root samples exhibited higher total phenolic and total flavonoid contents than most in vitro-derived tissues.
- Antioxidant activity did not show a direct relationship with total phenolic accumulation across plant parts.
- In vitro propagation and adventitious root culture demonstrated potential for conservation and sustainable utilization of medicinal plant resources.
- Biotechnology conservation may support long-term preservation and reduced harvesting pressure on natural populations.

1. Introduction

Mountain ecosystems of northern Pakistan represent important biodiversity hotspots and harbour numerous medicinally valuable plant species (Hamayun et al., 2003; Nasir & Ali, 1999). However, these ecosystems are increasingly threatened by climate change, rising temperatures, erratic rainfall patterns, habitat degradation, and overexploitation of natural resources. Such environmental disturbances are particularly alarming for indigenous medicinal plants because climatic variability can directly influence their growth, survival, and biosynthesis of therapeutically important secondary metabolites (Randriamampionona et al., 2007; Siddiqui et al., 2011). *I. heterantha,* a deciduous shrub of the family

Leguminosae, which is also frequently called Himalayan Indigo, is extensively spread throughout the tropical regions of the globe (Bakasso et al. 2008). In Pakistan, the genus *Indigofera* comprises about 24 species (Hamayun *et al*., 2003). *I. heterantha,* locally named as Ghorega (Hamayun et al., 2003), is used as folk medicine for the remedy of skin problems, abdominal pain, gastrointestinal disorders, spastic pain, and a number of infectious diseases in the Swat valley of Pakistan (Nasir and Ali, 1999). Presence of numerous phytochemicals like saponin, tannins, rutin, caffeic acid, galangin, quercetin, triterpenes, alkaloids, and steroids has been reported in the genus Indigofera (Rahman et al., 2005; Bakasso *et al*., 2008).

Bioactive compounds obtained from plants are widely used as medicines, and ongoing research is uncovering new plants and metabolites of interest. Though the quality and magnitude of such metabolites vary in plants available in their wild habitat, conventionally grown, or *in vitro* developed plants. The nature of these compounds depends on environmental factors such as soil, climate, and seasonal variations (Bhojwani & Razdan, 1996). Variation in bioactive metabolites among plant samples collected from plants grown under different climatic conditions has been reported previously (Randriamampionona et al., 2007). It is also evident that soil texture, pH, climatic conditions, and even different cultural practices may alter the bioactive constituents of medicinally important plants (Rouillard-Guellec et al., 1997; Siddiqui et al., 2011). In mountain ecosystems, environmental stresses often stimulate the accumulation of antioxidant compounds as adaptive responses to harsh ecological conditions. The increasing vulnerability of medicinal plants under changing climatic conditions has intensified the need for sustainable conservation and propagation strategies (Shaik et al., 2011). Excessive harvesting from natural habitats, rapid urbanization, and habitat fragmentation have placed several medicinal plant species at risk of population decline. In this context, plant tissue culture techniques provide an effective approach for ex situ conservation, mass propagation, and sustainable utilization of medicinal plants under controlled conditions (Jeyachandran et al., 2012). In vitro culture systems also provide opportunities to manipulate environmental and nutritional factors for enhanced biomass and secondary metabolite production. Parameters such as light quality, gaseous exchange, nutrient composition, and elicitor treatments can significantly influence phytochemical synthesis in cultured tissues (Berlin, 1986; Schlatmann et al., 1994; Zobayed et al., 2003). Despite the medicinal importance of *I. heterantha,* limited information is available regarding the comparative phytochemical potential of wild and in vitro propagated materials under mountain ecosystem conditions.

In this regard, strategies have been developed and implemented to increase the production of highly endangered medicinal plant species through *in vitro* propagation. Yet the medicinal potential of in vitro plants of *I. heterantha* has not been properly explored. Therefore, the current study aimed to compare the bioactive secondary metabolites of *I. heterantha* collected from natural habitats with those of plants produced under laboratory conditions via in vitro propagation. Mountain ecosystems are increasingly exposed to environmental changes that may affect the distribution and biochemical characteristics of medicinal plant species.

However, the present study did not directly evaluate climate-related variables. Therefore, this work focuses on comparing secondary metabolite accumulation and antioxidant potential between wild-grown and in vitro propagated materials of *I. heterantha*. The findings provide insights into the potential application of in vitro propagation and conservation biotechnology approaches for sustainable utilization and reduced harvesting pressure on natural populations.

2. Materials and Methods

2.1. Collection of plant material from wild habitat

The young, healthy leaves, flowers, and roots of *Indigofera heterantha* were collected from 3 locations in the Swat district (Madyan, Gharri, and Lalku) in May 2024 (Fig. 1) and carefully transported to the laboratory. Samples were dried in the shade prior to biochemical analyses.

Figure 1. Collection of several Parts of the *Indigofera heterantha* from the local Mountain

2.2. Preparation of sample extraction for analysis

The dried parts (shoots, leaves, and roots) taken from in vitro propagated plants and the plant parts collected from natural habitats, were crushed into powder by using a mortar and pestle for extract grinding. From the crushed powder of each sample, 10 mg was mixed with 10 ml of ethanol in the test tubes and placed in the dark. The prepared solutions of each sample were vortexed for 24 hours. After one week, the solutions were centrifuged for 15 minutes at 14,000 revolutions per minute. The extract was filtered through Whatman No. 1 filter paper and stored at 4 °C for further use in the experiment to quantify TPC, TFC, and DRSA.

2.3. Estimation of Total Phenolic Contents, Total Flavonoid Contents, and Analysis of Free Radical Scavenging Activity by DPPH

Each sample was analyzed to quantify TPC, TFC, and DPPH activities according to the protocols of Ahmad *et al.* (2014). According to the protocols of Ahmad *et al.* (2014) for TPC quantification, 0.1 ml Folin-Ciocalteus reagent (FCR), sample extract (0.03 ml), and 2.55 ml distilled water were mixed in a test tube and incubated for 30 minutes in dark conditions. The mixture of each sample was then centrifuged for 14 minutes at 10,000 rpm. Supernatant was taken in a cuvette and placed in a UV–visible spectrophotometer (Shimadzu-1650; Japan). The absorbance of the resulting mixture was set to 760 nm. Gallic acid (Sigma; 1.0–10 mg/ml; R2=0.9878) was used for plotting the custom standardization curve. From % TPC, results expressed as Gallic acid equivalents (GAE) mg/g of dry root biomass (DRB) were calculated using the following equation.

$$\text{Total phenolic content (\%)} = 100 \times (\text{AS-AB})/ (\text{CF} \times \text{DF})$$

Where, AS stands for absorbance of the sample, and AB represents absorbance of blank, CF is the conversion factor from the standard curve, and DF refers to the dilution factor.

Similarly, the TFC was calculated following the protocol of Ahmad et al. (2014), with slight modifications. Ethanolic extract (0.25 ml) of the under-trial samples was mixed with sterile distilled water (1.25 ml) and 0.075 ml 5% (w/v) $AlCl_3$. Before incubating for 5 minutes and centrifuging at 10,000 rpm for 14 min, the solution was further added with 0.5 ml of NaOH (1 M). The absorbance spectra were calculated at 510 nm with a UV–visible spectrophotometer (Shimadzu-1650PC, Japan). Rutin (Sigma; 1.0–10 mg/ml; R2=0.9866) was used for mapping the standard adjustment curve. The total flavonoid contents were displayed as rutin equivalent (RE) µg/g-DRB of extracts.

Each sample was further analyzed to quantify DPPH radical scavenging activity (DRSA). According to the method used by Ahmad *et al.* (2014), 1.0 ml of the ethanol extract from each sample was added to 2.0 ml of DPPH free radical solution (0.25 mg/ml × 4) and mixed uniformly using a vortex mixer. The prepared mixture was kept in the dark for the duration of 30 min. Using a visible spectrophotometer at room temperature, the absorbance of the resulting mixture was calibrated at 517 nm. Lastly, the radical scavenging activity was calculated as a percentage of DPPH discoloration via the following equation.

$$\text{DRSA (\%)} = 100 \times (1 - \text{AP/AD})$$

Where AP symbolizes the absorbance of the plantlet extract at 517 nm, and AD displays the absorbance of the DPPH solution without tissue extract.

Statistical Analysis

Replicated mean values and least significant difference (LSD) tests were analyzed using Statistix software (version 8.1) following the protocols of a Completely Randomized design, and for graphical demonstration, Origin Lab (version 8.5) software was used.

3. Results and Discussion

3.1. Total phenolics and flavonoids (µg g^{-1} DW) accumulation

The results revealed significant variation in phenolic and flavonoid accumulation among different plant parts of wild-grown and in vitro propagated *I. heterantha*. Generally, higher concentrations of secondary metabolites were recorded in plant materials collected from natural mountain habitats compared with in vitro propagated tissues (Figs. 2 & 3). The contents of total phenols (3623.9 µg g^{-1} of DW) and flavonoids (468.9 µg g^{-1} of DW) were higher in the root samples collected from the wild habitat. However, the *in vitro* adventitious roots yielded comparatively maximum quantity of TPC (2873.9 µg g^{-1} DW) and TFC (360.8 µg g^{-1} DW) after the wild plant's roots, followed by the root extract of the *in vitro* induced roots that yielded TPC (1420.2 µg g^{-1} DW) and TFC (308.3 µg g^{-1} DW). There were no significant differences in the content of total phenols and total flavonoids among the ethanolic extracts of micropropagated shoots (114.6 µg g^{-1} DW and 201.2 µg g^{-1} DW), wild collected leaves (65.6 µg g-1 DW and 191.1 µg g-1 DW), and flower samples having phenolic contents of 85.0 µg g^{-1} DW. Flowers collected from wild plants of *I. heterantha* yielded the lowest total flavonoid content (15.7 µg g-1 DW) (Fig. 2). Secondary metabolites present in plants enhance the medicinal value of plants as reported earlier by Randriamampionona et al., (2007). The findings showed that micro-propagated plants have lower concentrations of the bioactive compounds (TPC and TFC) than plant samples collected from wild habitats. Several

factors like environmental conditions, genetic diversity and nutrients present in the soil may cause variations in concentration of bioactive compounds (Bourgaud et al., 2001). Differences in the production of bioactive chemicals among tissues may be due to differences in tissue nature, endogenous hormone levels, and the physiological condition of plant parts (Chavan et al., 2014). Although nutrient availability and growth conditions were highly conducive to callus induction, significant variation was observed in the synthesis of organic chemicals between wild and in vitro plants. Variations in nutrient concentrations, light intensity, and rhizosphere microbial activity may affect plant cell growth and, therefore, their metabolic functions (Rout et al., 2006; Barik et al., 2007). These findings suggest that environmental variability associated with mountain ecosystems strongly influences phytochemical biosynthesis in *I. heterantha*. The study further demonstrates that conservation biotechnology could serve as a climate-resilient strategy for preserving medicinal plant resources threatened by changing climatic conditions.

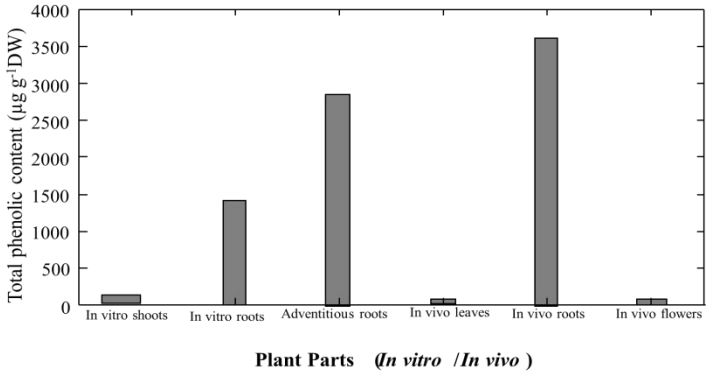

Figure 2: Total phenolic contents (µg g^{-1} DW) of *in vitro* and wild collected plant parts of *Indigofera heterantha.*

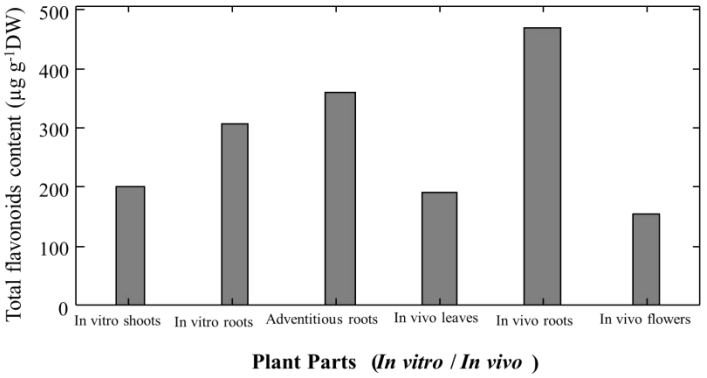

Figure 3: Total flavonoid contents (µg g^{-1} DW) of in vitro and wild-collected plant parts of *I. heterantha.*

3.3. DPPH free radical scavenging assay

The antioxidant potential of the different extracts was confirmed using the DPPH radical-scavenging assay (DRSA). This assay has been extensively used to examine the antioxidant properties of various medicinally important plant extracts. All analyzed extracts showed substantial differences in DPPH radical-scavenging activity among the collected samples (Fig. 4). The highest DPPH scavenging activity (63.4%) was observed in the leaves of *I. heterantha* collected from its natural habitat (Fig.1). The *in vitro* induced roots were found with DRSA (46.7%). The *in vitro* grown shoots exhibited moderate (DRSA; 38.1 %). The statistically similar DRSA (35.0 and 33.7 %) was estimated in the extracts of *In vitro* adventitious roots and the flowers gathered from *ex vitro*. Furthermore, the lowest DPPH radical scavenging (30.6%) was recorded in the wild-collected root of *I. heterantha.*

DPPH is a purple-coloured stable organic radical, which upon accommodating an electron from an antioxidant becomes yellow, which can be read spectrophotometrically. Hence, the quantity of antioxidants in the reaction mixture can be estimated by the degree of discoloration (Chavan *et al*., 2014). The DPPH assay provides quantitative data on the reactivity of the test compounds with stable free radicals. DPPH testing is widely used to assess antioxidant capacity by scavenging free radicals from the samples under investigation (Devendra et al., 2012).

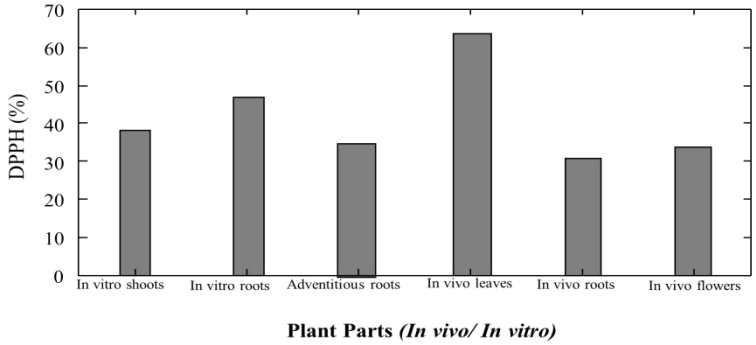

Figure 4. DPPH free radical scavenging activities (%) of *in vitro* and wild collected plant parts of . *heterantha*.

Current research has shown that micropropagated roots exhibited greater scavenging potential than other in vivo and in vitro plant extracts, except for the wild-collected leaf extract. *In vitro* cells are in a rapidly proliferating stage, so they accumulate more sugars as they are readily available in the culture medium. A similar pattern of antioxidant potential has been confirmed by Chauhan and Vimala. (2009), who compared *in vitro* and *in vivo* biochemical performance in *Adhatoda vasica.*

The present findings support the concept that climate-induced environmental stresses significantly influence phytochemical and antioxidant responses in medicinal plants. Consequently, integrating tissue culture approaches with conservation strategies could contribute towards climate-resilient medicinal plant production and sustainable mountain agriculture.

Conclusion

The present study demonstrated considerable variation in phytochemical accumulation and antioxidant activity between wild and in vitro propagated *Indigofera heterantha.* Micropropagated roots exhibited maximum total phenolic and flavonoid contents, while leaves collected from natural mountain (wild) habitats displayed the highest antioxidant activity. Environmental conditions associated with mountain ecosystems appear to play a significant role in regulating secondary metabolite biosynthesis. Abiotic stresses prevalent in natural habitats may stimulate enhanced accumulation of phenolics and flavonoids as adaptive responses. Although comparatively lower metabolite concentrations were observed in *in vitro* propagated materials, adventitious root cultures showed promising potential for sustainable production of bioactive compounds. Therefore, plant tissue culture techniques could provide an effective climate-resilient strategy for conservation, propagation, and sustainable utilization of medicinal plants threatened by climate change and habitat degradation.The study highlights the importance of integrating conservation biotechnology and in vitro propagation systems for sustainable mountain agriculture and long-term preservation of Himalayan medicinal plant resources.

Recommendations

- Adventitious root cultures of *Indigofera heterantha* should be supplemented with suitable biotic and abiotic elicitors to enhance secondary metabolite production.
- Further investigations should evaluate the impact of climate-related stresses, such as drought and temperature fluctuations, on phytochemical biosynthesis.
- Biotechnology approaches should be integrated into medicinal plant conservation programs in mountain ecosystems.
- Large-scale in vitro propagation systems may provide sustainable alternatives to the overharvesting of medicinal plants from natural habitats.
- Future studies should focus on metabolomics profiling and molecular characterization of stress-responsive pathways associated with climate adaptation.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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