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Effects of Protein Hydrolysates and Seaweed Extract Application on some Morphological Parameters, Phytochemicals, and Antioxidant Capacity of Violet (*Viola ignobilis* Rupr.) under Two Light Intensities

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ABSTRACT

Domestication of medicinal plants through sustainable agricultural practices represents a novel challenge in the field of medicinal plant production. This study aimed to assess the effects of light intensity and biostimulant application on the growth and biochemical composition of *Viola ignobilis* Rupr. To this end, plants were cultivated under two light levels (50% and 100% of full natural irradiance) and treated with various biostimulants: animal-derived protein hydrolysate (A-PH), vegetal-derived protein hydrolysate (V-PH), seaweed extract (SWE), as well as combinations of A-PH + SWE and V-PH + SWE, with water serving as the control. Both light intensity and biostimulant application had significant effects on morphological parameters, including the fresh and dry weight of aerial parts, as well as leaf length and width, though their interaction only influenced leaf area. Maximum leaf fresh weight and length were observed in plants exposed to 100% light intensity, while the greatest leaf width and area were recorded in those grown under 50% light intensity. Furthermore, the results indicated that total phenol and flavonoid contents were markedly higher at 100% light intensity compared to shaded plants. Additionally, plants treated with biostimulants exhibited significantly enhanced phenol and flavonoid levels relative to the control. Antioxidant activities also increased under 100% light intensity. Overall, the combined application of PHs and SWE, due to synergistic effects, led to improvements in the parameters studied, while full irradiance enhanced the phytochemical content and antioxidant potential of *Viola ignobilis*. This work demonstrates that optimizing cultivation techniques through eco-friendly approaches can enhance crop performance and phytochemical content in violet, especially in the absence of conventional fertilizers.

Introduction

Viola ignobilis Rupr., commonly known as violet, is a valuable medicinal herb belonging to the Violaceae family. It is widely used in Iranian traditional medicine to treat ailments such as sore throat, asthma, common cough, dyspnea, bronchitis, tonsillitis, and pneumonia (Feizabadi

et al., 2017). All parts of the plant have medicinal applications (Ghasemzadeh et al., 2015). Additionally, violet is well-regarded for its pharmaceutical properties within the Ayurvedic and Unani medicinal systems (Mittal et al., 2015). The plant is rich in mucilage, methyl salicylate, glycosides, saponins, alkaloids, tannins, cyclotides, as well as phenolic and flavonoid compounds (Kundal et al., 2022). In Iran, *Viola ignobilis* is one of the important medicinal species found in the Arsbaran region in the northwest. However, the species is endangered due to overharvesting in its natural habitats. This underscores the necessity of domestication and cultivation of the plant in medicinal farms. Proper growth conditions are critical to enhancing the growth and performance of plants during the domestication process (Hamidah et al., 2018).

Research has demonstrated that *Viola ignobilis* possesses potent antioxidant properties (Ebrahimzadeh et al., 2010). In contemporary times, the human body is increasingly exposed to free radicals that cause significant damage to lipids, proteins, and DNA, which can trigger carcinogenesis, inflammatory, and cardiovascular diseases (Lobo et al., 2010). Furthermore, the use of synthetic antioxidants has been reported to pose risks to human health (Petcu et al., 2023). Consequently, there is a growing global trend towards utilizing natural antioxidants derived from plants (Anbudhasan et al., 2014). Phytochemicals, the primary source of antioxidants, play a crucial role in mitigating the damaging effects of oxidative stress and other adverse cellular responses (Engwa, 2018). A substantial portion of the antioxidants found in plants are products of secondary metabolism (Rajashekar et al., 2009). Various factors, including genotype, growth stage, biotic and abiotic influences, as well as crop management practices, can affect phytochemical levels in plants (Biondi et al., 2021).

Studies have shown that the accumulation of phytochemicals in response to environmental conditions has been extensively studied across a wide range of plant species. Phenolic and flavonoid contents in medicinal plant extracts serve as cost-effective antioxidants by inhibiting free radical formation and preventing auto-oxidation (Devequi-Nunes et al., 2018). Recent studies highlight that environmental factors and cultivation techniques significantly influence phytochemical accumulation and antioxidant potential in medicinal herbs (Li et al., 2020; Chen et al., 2018; Kaunda et al., 2018; Grulke and Heath, 2020). Under different environmental conditions, the production of secondary metabolites can increase or decrease by up to 50% (Pant et al., 2021). Therefore, optimizing cultivation techniques is vital for increasing yield and enhancing the medicinal value of plants.

Light is an ecologically limiting factor that affects both plant growth and the accumulation of secondary metabolites (Li et al., 2020; Thoma et al., 2020; Hashim et al., 2021). Determining the optimal light conditions for phytochemical biosynthesis is crucial to obtaining the maximum concentration of bioactive compounds in medicinal plants (Marchant et al., 2022). Beyond light intensity, the application of plant biostimulants represents a new eco-friendly approach to improving the synthesis and accumulation of secondary metabolites, a topic that has attracted considerable research interest. Biostimulants are biological compounds that enhance crop yield, improve quality, and increase tolerance or mitigate adverse impacts caused by stress (Sun et al., 2024). Previous studies suggest that biostimulants play several roles in promoting plant growth and development by influencing physiological processes (Yuan and Dickinson, 2023; Elwaziri et al., 2023; Munaro et al., 2024). These compounds also enhance plant resistance to a broad range of biotic and abiotic stresses (Vaseva et al., 2022; Francesca et al., 2022). Moreover, the use of biostimulants can reduce or eliminate the need for chemical fertilizers (Moreno-Hernandez et al., 2019). Previous research has demonstrated the efficacy of biostimulants in enhancing the phytochemical and nutritional value of various plants (Zhou et al., 2022; Tallarita et al., 2023). Multiple studies have confirmed the effectiveness of biostimulant application in promoting secondary metabolite biosynthesis. For example, Szczepanek et al. (2020) reported that Kelpak seaweed extract increased the accumulation of bioactive compounds, including polyphenols, chlorogenic acid, and flavonoids, in carrots. Additionally, Abeed et al. (2021) found that the phenolic compounds in *Catharanthus roseus* were significantly enhanced by applying leaf extract from *Calotropis procera* as a biostimulant.

Protein hydrolysates (PHs) consist of oligopeptides, polypeptides, and free amino acids, which can be derived from vegetal or animal agro-industrial byproducts through chemical or enzymatic hydrolysis (Rouphael and Colla, 2020). Recent evidence suggests that PHs can induce hormone-like activity (Colla et al., 2014) and enhance nutrient uptake (Ceccarelli et al., 2021), thereby promoting plant growth under both adverse and normal environmental conditions. SWE represent another category of biostimulants, comprising polysaccharides, alginates, polyphenols, betaines, amino acids, and vitamins.

Additionally, SWE contain phytohormones such as auxin, abscisic acid, and cytokinins, which may influence physiological and biochemical processes in plant cells (Baltazar et al., 2023).
The interactions between light intensity and plant biostimulants have been scarcely studied, highlighting a gap in the current research. Therefore, this study aimed to evaluate the effects of modulating light intensity and the application of three different biostimulants—seaweed extract, vegetal-based protein hydrolysate, and animal-based protein hydrolysate—on the morphological and biochemical traits of *Viola ignobilis* Rupr.

Materials and Methods

Plant collection

The experiment was carried out from 15 January to 15 April 2021 on a farm located in Roudsar, a city in Guilan province in northern Iran (37° 08' 15.40" N, 50° 17' 16.80" E, 2 m a.s.l). Seedlings of violet were collected from Kaleybar County, East Azarbaijan, Iran (38° 51' 59.99" N., 47° 01' 60.00" E., 1144 m a.s.l) on 10 December. The identification of species (*Viola ignobilis* Rupr.) was confirmed by the Guilan Agriculture and Natural Resources Research Center. Four-leaf stage seedlings were transplanted in December 2020 into plastic pots filled with a mixture of forest soil and leaf mold with equal proportions. The final substrate had a pH of 7.35 and an EC of 1.08 dS m^{-1}. The soil was sandy loam (75% sand, 17% silt, 8% clay), with organic matter of 10%, total nitrogen of 3.1%, available P at 10 mg kg^{-1}, K at 145.21 mg kg^{-1}, Fe 10 mg kg^{-1}.

Shade treatments

Plants were randomly divided into two groups which were subjected to two different light intensities. The mean daily variation in full sunlight from January to April measured by using a HT620 Digital Lux Meter (Habotest, China). In order to measure light changes, light intensity was measured three times a d at 10 am, 12 noon and 2 pm, and at the end of each month, the average light intensity was recorded (Fig. 1). Shade treatment was performed using green shading net cloth 50% was made with high-density polyethylene plastics above the wooden frames and fixed at a height of 3 m above the ground to provide a 50% decrease in natural light intensity (Shao et al., 2014).

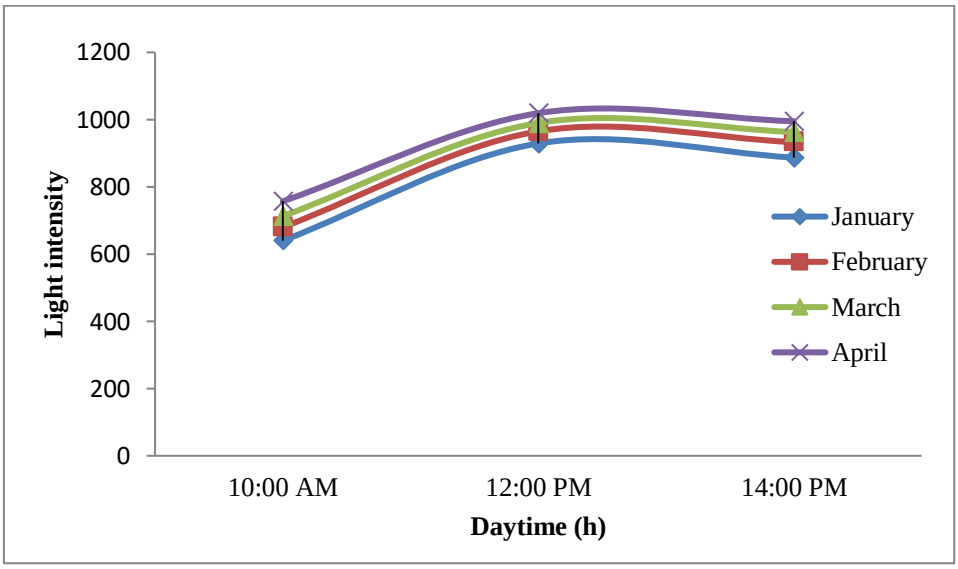

Fig. 1. Variations in light intensity (µmol m^{-2} s^{-1}) in open field (100% full irradiance). Data were measured three times a d at 10 am, 12 noon and 2 pm, and at the end of each month, the average light intensity was recorded. Each point on the curve is the average for one month at that h.

Biostimulants applications

The PHs were applied by foliar spray at the concentration of 0.2 g L -1 (Cristiano et al., 2018) on the leaves of violet weekly on January 15 and continued for 12 week. The extract of *Ascophyllum nodosum* (Acadian Plant Health, Canada) contains amino acid 4.4%, mannitol 4%, alginic acid 10%, and other organic compounds 55%. The elemental composition of Acadian as follows: N 1.5%, K 17%, P 0.2%, sulphur 1%, Mg 0.3%, Ca 0.4%, Fe 150 ppm. This compound was applied directly to the soil by fertigation method (500 mL per pot) at 2 g L^{-1} every two weeks from the third week of seedling cultivation for 3 months. The treatment application continued until the flowering stage. No fertilizer was utilized, and crop management was performed the following standard methods. The animal-

protein hydrolysate was used in this work, obtained from the enzymatic hydrolysis of fish in alkaline conditions (Madende and Hayes, 2020) and the vegetal-protein hydrolysate obtained through enzymatic hydrolysis of soybean seeds (Barrada et al., 2022).

Plant measurements

Morphological parameters

At the end of the flowering stage (121 d after cultivation), the leaves of violet were separated from the roots. The morphological analysis of aerial parts was achieved using fresh and dry weight and also the length, width, and leaf area. The aerial parts were dried in an oven at a temperature of 70 °C for 72 h to reach a constant weight. The aerial fresh and dry weight was measured by laboratory digital scale. The length and width of leaves determined by ruler. Also the total leaf area per plant was measured by a leaf area meter (Delta-T, Decagon Devices, Pullman, Washington, USA).

Determination of total phenolic compounds (TPC)

The total phenolic compound of violet was determined according to the procedure used by Singleton et al. (1999). To 0.5 mL of the violet extract, 0.5 mL distilled water and 2 mL Folin–Ciocalteau reagent was added and the prepared mixture was incubated for 10 min in a dark room. Then, 10 mL of 20% (w/v) sodium carbonate was added to the mixture and the final content was incubated in the dark condition at room temperature for 30 min. finally, the absorbance of samples was read at 725 nm by spectrophotometer. Results are expressed as milligrams of Gallic acid equivalent per gram dry weight.

Total flavonoid content (TFC)

The total flavonoid content of violet was measured by the aluminum chloride spectrophotometric method which is one of the most common procedures described by Zhishen et al. (1999). In this method, 0.5 mL of prepared methanolic extract was mixed with 150 µL of 15% sodium carbonate solution, then after 6 min of incubation in a dark room, 150 µL of 10% aluminum chloride was added to the mixture and then incubated for 6 min again. Finally, 2 mL of 4% sodium hydroxide and 2 mL of distilled water were added and incubated for 10 min at room temperature. The absorbance of samples was determined by spectrophotometer at 510 nm. Results were expressed as milligrams of Quercetin equivalent per gram dry weight.

Total anthocyanin content

Total anthocyanin was determined according to the method used by Wagner (1979). One g of fresh leaf of sweet violet was homogenized in 10 mL of acidified methanol (Methanol: HCl 99: 1 v/v) and maintained for 24 h in dark condition at room temperature. Then, the extract was centrifuged at 4000 g for 10 min at room temperature. The absorbance of each supernatant was read at 550 nm using spectrophotometer. The extinction coefficient 33,000 (mM^{-1} cm^{-1}) was used to determine the total anthocyanin concentration which expressed as µmol g^{-1} fresh weight.

Determination of free radical scavenging activity

DPPH

The DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay was used according to Brand-Williams et al. (1995) to evaluate the free radical scavenging activity of violet extracts. To this end, 80 µL of methanolic extracts were mixed with 1.92 mL of DPPH solution. Then, after 2.5 min of incubation absorbance of samples was read at 515 nm. The affinity of the test material to quench DPPH free radicals was calculated according to the following equation:

$$Scavenging\% = \frac{100 \times (A0 - As)}{A0}$$

Which in this equation, A0 = absorbance of control at 0 min, As = absorbance of sample.

Assessment of antioxidant capacity by the $ABTS^{+\bullet}$ cation radical method

ABTS+ radical scavenging activities of leaf extract of violet (Viola ignobilis Rupr.) were done according to the procedure of Re et al. (1999). The first step was to produce the $ABTS^{+\bullet}$ cation radical using the incubation of 7 mM ABTS [(2,29-azinobis-(3-ethyl benzothiazoline-6-sulfonic acid)] solution and 2.45 mM potassium persulfate (K2S2O8) in a ratio of 1:0.5. The prepared solution was left in a dark condition for 12 h at room temperature. Before to the measurement, the $ABTS^{+\bullet}$ solution was diluted with phosphate-buffered saline with a pH of 7.4 (PBS) to obtain the absorbance value of 0.70 ± 0.020 at 734 nm as a stock standard. Then, 50 µL of samples were added to 5 mL of diluted $ABTS^{+}$ solution. Finally, the obtained mixture was shaken and then placed in a water bath at 30 °C for 6 min, following the absorbance of samples was measured using a spectrophotometer at 734 nm.

Determination of antioxidant capacity (FRAP)

The antioxidant capacity was evaluated via Ferric Reducing Ability of Plasma (FRAP) method using a procedure described by Benzie and Strein (1996). For reagent preparation, 250 mL acetate buffer (pH = 3.6), 25 mL TPTZ solution in 40 mL HCl and 25 mL of FeCl3.6H2O (20 mM) were mixed. The FRAP reagent was warmed to 37 °C, then 6 mL of solution was added to 200 µL of samples and 600 µL H_2O. The absorbance of the final dilution of sample was read at 593 nm.

Experimental design and statistical analysis

The experimental design was a split-plot arrangement based on randomized complete blocks with three replicates. In this work, two light regimes consisting of 50 and 100% full natural irradiance as the main factor and the biostimulant application including animal-protein hydrolysate (A-PH), vegetal-protein hydrolysate (V-PH), seaweed extract (SWE), and the combination of A-PH + SWE and V-PH + SWE as sub-factors were assessed. Plants treated with H2O were used as control. Data Analysis was done using the ANOVA procedure in SAS version 9.2 (SAS Ins., Cary, NC, USA). Differences between treatment means were achieved by the least significant difference (LSD) ($P \leq 0.05$).

Results

Leaf morphological parameters

Both light intensity and biostimulant application had a statistically significant effect ($P \leq 0.01$) on the aerial fresh weight of plants (Table 1). However, the interaction between these two factors did not yield a significant impact on this parameter. Plants exposed to 100% light intensity exhibited a 10% increase in fresh weight compared to those grown under shaded conditions (Fig. 2). Additionally, plants treated with the A-PH + SWE combination achieved the highest fresh weight (42.65 g), although no significant difference was observed between the A-PH + SWE and V-PH + SWE treatments. Conversely, the lowest aerial fresh weight (19.18 g) was recorded in untreated plants (Fig. 3).

Table 1. Variance analysis of light intensity, biostimulant and their interaction (mean of squares) on some morphological parameters of *Viola ignobilis* Rupr.

		MS				
S.O.V	**DF**	**Leaf area**	**Leaf length**	**Leaf width**	**Aerial fresh weight**	**Aerial dry weight**
Block	2	1.9687^{ns}	2.58967^{*}	1.79388^{ns}	5.59^{ns}	0.3238^{ns}
Light	1	24.3049^{**}	7.4529^{**}	7.756225^{**}	47.90946^{**}	0.880469^{ns}
Light × block	2	0.83430^{ns}	2.93290^{**}	3.179758^{**}	4.40^{ns}	0.976452^{ns}
Biostimulants	5	153.8763^{**}	3.11064^{**}	4.249962^{**}	420.1^{**}	47.25635^{**}
Biostimulants × light	5	3.48541^{*}	0.11179^{ns}	11.443011	2.948^{ns}	0.144742^{ns}
Experimental error	20	24.8825	11.44301	50.77383	62.5622	23.4292
Total	35	-	-	-	-	-
C.V.%	-	4.92	15.96	13.57	5.15	19.14

S.O.V.: Source of variation, DF.: Degree of freedom, M.S.: Mean squares, CV: Coefficient of variation. **: significance at $P \leq 0.01$, *: significance at $P \leq 0.05$, NS: No Significance.

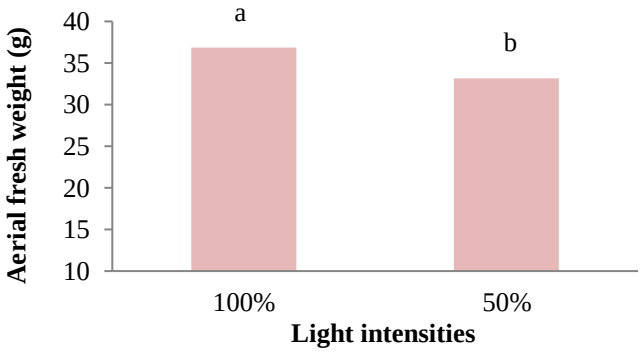

Fig. 2. Simple effect of light intensity on aerial fresh weight per plant. Different letters on each bar indicate significant differences according to the least significant difference (LSD) ($P \leq 0.05$).

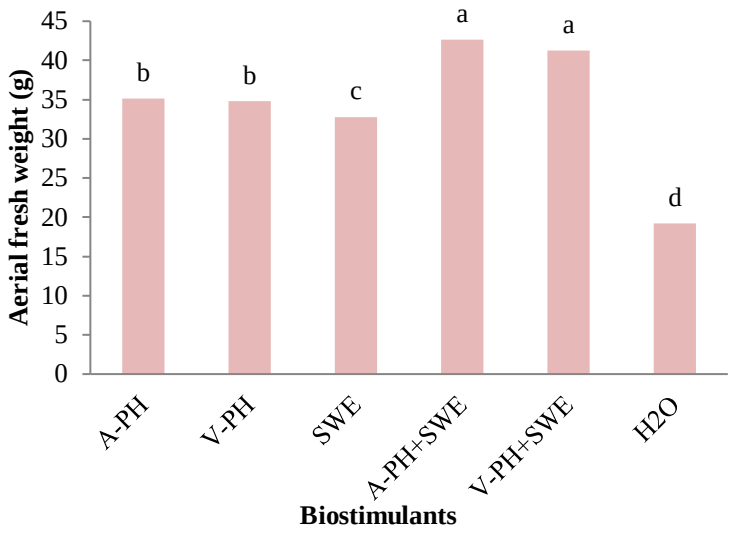

Fig. 3. Simple effect of biostimulants application on aerial fresh weight per plant. Animal protein hydrolysate (A-PH), vegetal protein hydrolysate (V-PH), seaweed extract (SWE), plants treated with H_2O served as a control. Different letters on each bar indicate significant differences according to the least significant difference (LSD) ($P \leq 0.05$).

The results presented in Table 1 indicate that both light intensity and biostimulant application had a significant effect ($P \leq 0.01$) on the dry weight of aerial parts. However, the interaction between these two factors did not exert a significant influence on this parameter. Specifically, the dry weight of aerial parts in violets increased by 29.39% under 100% light intensity compared to 50% light intensity (Fig. 4). The highest dry weight of aerial parts (8.52 g) was observed in plants treated with the A-PH + SWE combination, though no statistically significant difference was found between A-PH + SWE and V-PH + SWE treatments. Conversely, the lowest leaf dry weight (2.37 g) was recorded in the control plants (Fig. 5).

As can be seen in Table 1, the interaction between light intensity and biostimulant application had a significant impact ($P \leq 0.05$) on violet leaf area. The higher leaf area (26 cm2) in 100% light intensity was recorded in plants treated with A-PH + SWE without any significant difference with V-PH + SWE. A lower leaf area (12.4 cm^2) was observed in control plants. In the 50% light intensity, a higher leaf area (30.4 cm2) occurred in response to A-PH + SWE. A lower leaf area (14.5 cm^2) occurred in untreated plants at 100% light intensity (Fig. 6).

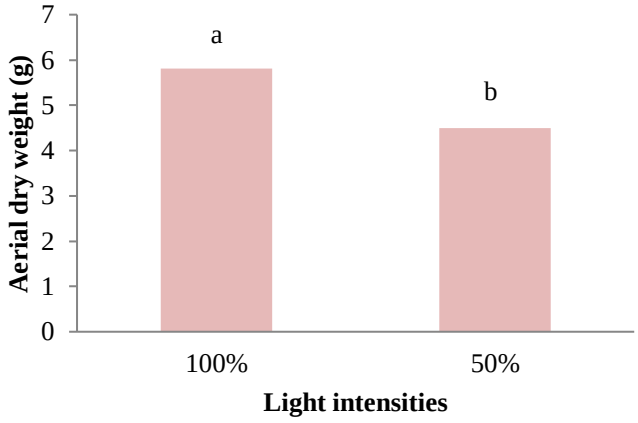

Fig. 4. Simple effect of light intensity on aerial dry weight per plant. Different letters on each bar indicate significant differences according to the least significant difference (LSD) ($P \leq 0.05$).

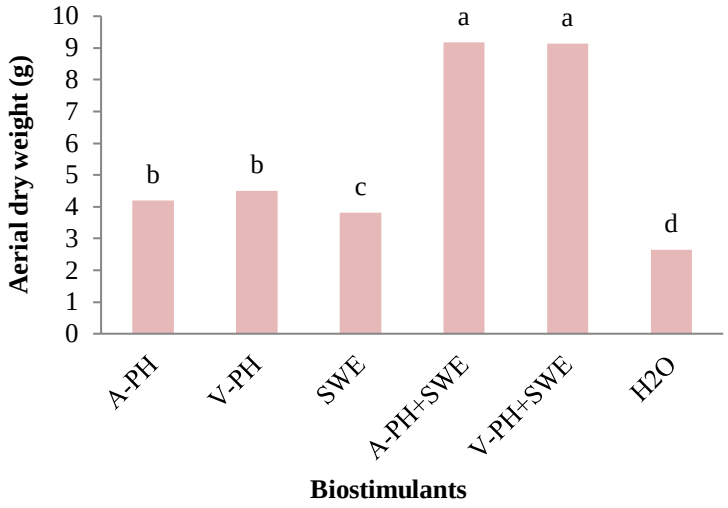

Fig. 5. Simple effect of biostimulants application on aerial dry weight per plant. Animal protein hydrolysate (A-PH), vegetal protein hydrolysate (V-PH), seaweed extract (SWE), plants treated with H_2O served as a control. Different letters on each bar indicate significant differences according to the least significant difference (LSD) ($P \leq 0.05$).

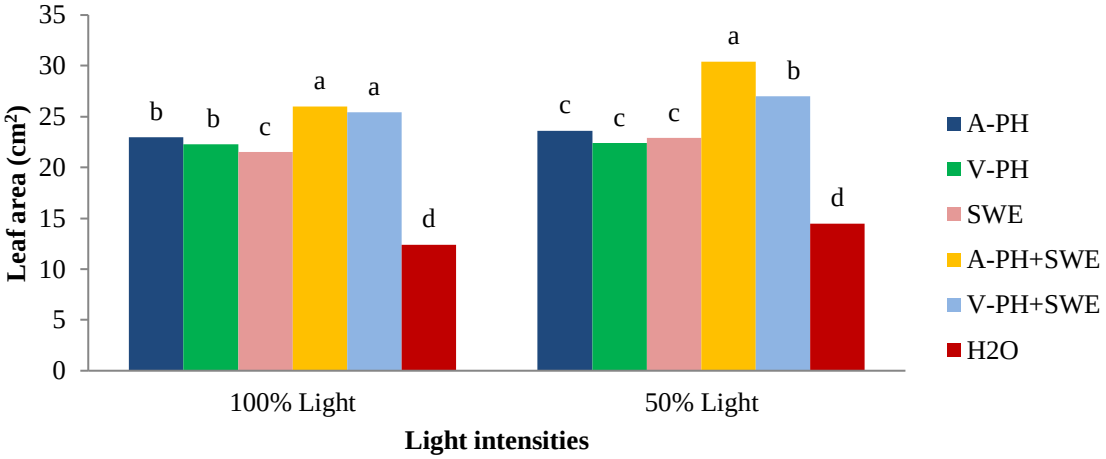

Fig. 6. The interaction effect of light intensity and biostimulant application on leaf area (for one leaf). Animal protein hydrolysate (A-PH), vegetal protein hydrolysate (V-PH), seaweed extract (SWE), plants treated with H2O served as a control. Different letters on each bar indicate significant differences according to the least significant difference (LSD) ($P \leq 0.05$).

Leaf length was significantly influenced by both light intensity and biostimulant application ($P \leq 0.01$), though their interaction did not yield a significant effect on this trait (Table 1). Leaf length increased by 18% in full sunlight compared to shaded conditions (Fig. 7). Additionally, biostimulant application had a notable impact on leaf length. The maximum leaf length (6.43 cm) in violets was observed in plants treated with A-PH + SWE, though no significant differences were found between the A-PH + SWE, V-PH + SWE, and A-PH treatments. In contrast, the shortest leaf length (4.5 cm) was recorded in untreated plants (Fig. 8).

In this experiment, leaf width in violets was significantly influenced by both light intensity and biostimulant application ($P \leq 0.01$), while the interaction between these factors did not have a significant impact on this trait (Table 1). As illustrated in Figure 9, shading resulted in a 17.64% increase in leaf width compared to plants grown in full sunlight. The highest leaf width (6.3 cm) was observed in plants treated with A-PH + SWE, although no significant differences were detected among the A-PH + SWE, V-PH + SWE, A-PH, and V-PH treatments. Conversely, the smallest leaf width (4 cm) was recorded in untreated plants (Fig. 10).

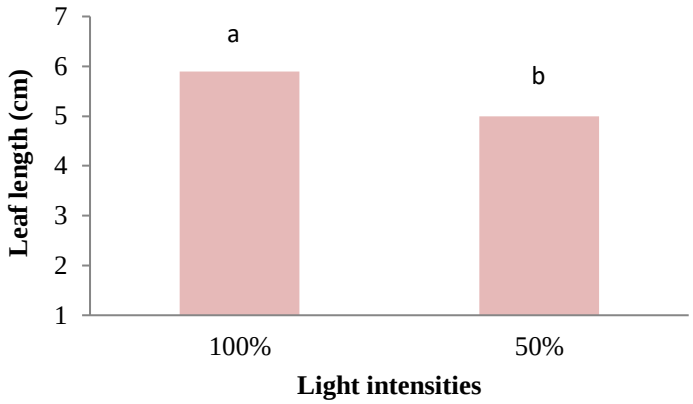

Fig. 7. Simple effect of light intensity on leaf length (cm) for one leaf. Different letters on each bar indicate significant differences according to the least significant difference (LSD) (P≤0.05).

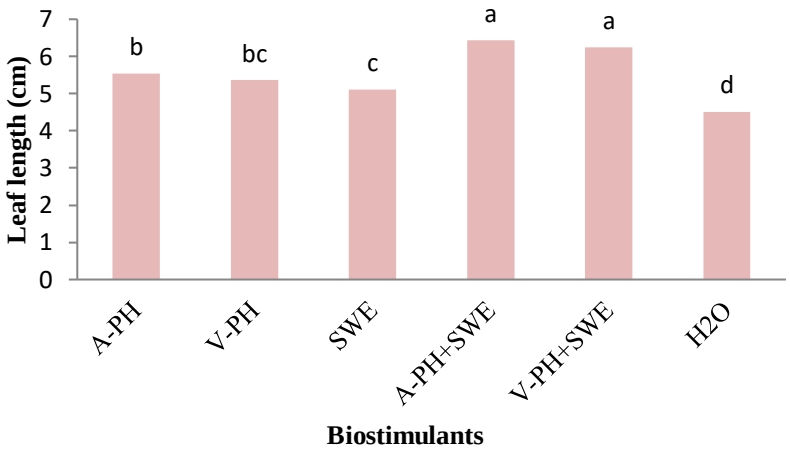

Fig. 8. Simple effect of biostimulants application on leaf length (cm) for one leaf. Animal protein hydrolysate (A-PH), vegetal protein hydrolysate (V-PH), seaweed extract (SWE), plants treated with H2O served as a control. Different letters on each bar indicate significant differences according to the least significant difference (LSD) (P≤0.05).

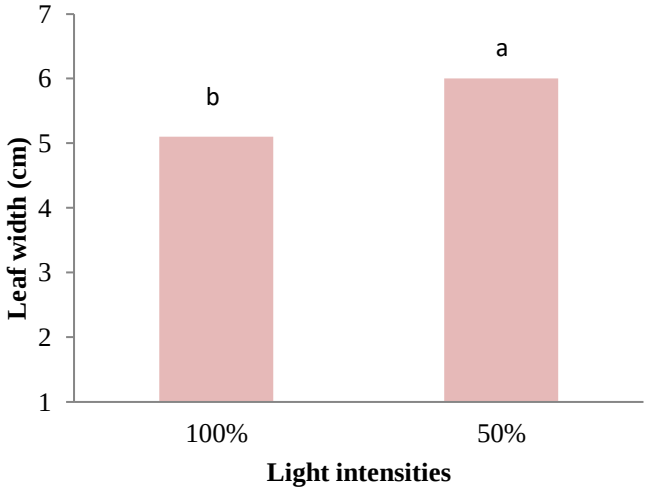

Fig. 9. Simple effect of light intensity on leaf width (cm) for one leaf. Different letters on each bar indicate significant differences according to the least significant difference (LSD) (P≤0.05).

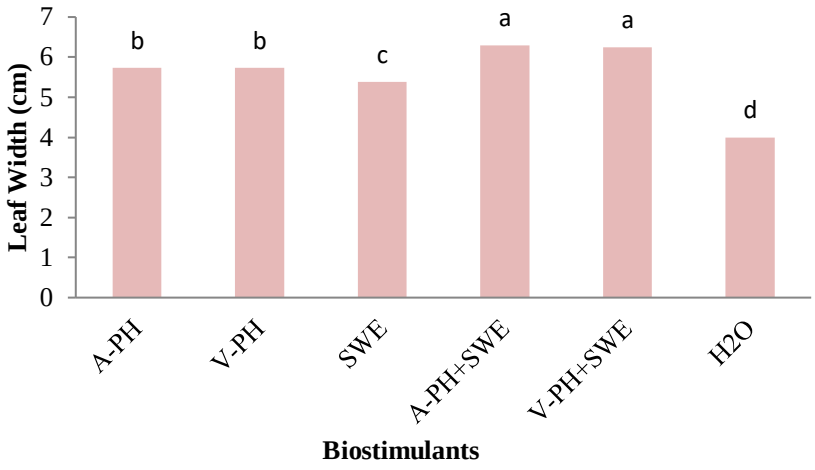

Fig. 10. Simple effect of biostimulants application on leaf width (cm) for one leaf. Animal protein hydrolysate (A-PH), vegetal protein hydrolysate (V-PH), seaweed extract (SWE), plants treated with H2O served as the control. Different letters on each bar indicate significant differences according to the least significant difference (LSD) (P≤0.05).

Phytochemical and antioxidant activity

The simple impact of light intensity and the biostimulant treatment had a significant influence on the total phenolic contents of the leaf (P≤0.01), but, no significant difference was observed between the interaction light intensity and the biostimulants (Table 2). The total phenolic concentration was highest by 10.17% in plants grown in full irradiance rather than shade treatment. Also, the highest total phenolic concentration (56.7 mg GAE g^{-1} DW) was recorded in treated plants with V-PH + SWE, but no significant differences were found between A-PH + SWE and A-PH + SWE, furthermore, the lowest total phenolic content (36.68 mg GAE g^{-1} DW) was observed in control plants (Table 3).

Table 2. Variance analysis of light intensity, biostimulant and their interaction (mean of squares) on some phytochemical traits of *Viola ignobilis* Rupr.

		MS					
S.O.V	**DF**	**Total Phenolic Compound**	**Total Flavonoid Compound**	**Anthocyanin**	**DPPH**	**ABTS**	**FRAP**
Block	2	13.99860^{ns}	6.064158**	0.005486^{ns}	0.329425*	121.02027**	0.329425*
Light	1	209.573878**	91.266178**	0.156025**	0.632025*	513.7777**	0.632025*
Light × Block	2	19.29021^{ns}	0.251103^{ns}	0.043275^{ns}	0.056758^{ns}	9.52694^{ns}	0.056758^{ns}
Biostimulants	5	360.137413**	214.87889**	0.482636**	1.394698**	611.16977**	1.394698**
Biostimulants × Light	5	0.734491^{ns}	0.472698^{ns}	0.011311^{ns}	0.037378^{ns}	4.08977^{ns}	0.037378^{ns}
Experimental error	20	311.032344	14.30574	0.857077	1.9717	322.96555	1.9717
Total	35	-	-	-	-	-	-
C.V.%	-	7.59	2.26	3.58	9.75	6.26	9.75

S.V.: Source of variation, d.f.: Degree of freedom, M.S.: Mean squares, CV: Coefficient of variation. **: significance at $P \leq 0.01$, *: significance at $P \leq 0.05$, NS: No Significance.

As shown in Table 2, the total flavonoid concentration in violet leaves was significantly influenced by both light intensity (P≤0.01) and biostimulant treatment (P≤0.01), though their interaction did not yield a significant effect. Specifically, light intensity positively increased total flavonoid content by 10.15% compared to shaded plants. Additionally, biostimulant-treated plants exhibited a substantial increase in flavonoid content compared to untreated plants.

The highest total flavonoid content (41.14 mg QE g-1 DW) was observed in plants treated with V-PH + SWE, though no significant differences were found between the V-PH + SWE and A-PH + SWE treatments. The lowest concentration (25.72 mg QE g^{-1} DW) was recorded in untreated plants (Table 3).

Table 3. Comparison of mean values for some phytochemical properties and antioxidant activity in violet (*Viola ignobilis* Rupr.).

Treatments	Phytochemical parameters					
	Total Phenol (mg GAE g^{-1} DW)	Total Flavonoid (mg QE g^{-1} DW)	Total Anthocyanin (µmol g^{-1} FW)	DPPH (%)	ABTS (%)	FRAP (mmol Fe^{+} g^{-1})
Light Intensity						
L1	54.366^{a}	38.94^{a}	0.0853^{a}	53.89^{a}	67.88^{a}	3.35^{a}
L2	49.341^{b}	35.35^{b}	0.0779^{b}	47.85^{b}	60.33^{b}	3.08^{a}
Biostimulants						
A-PH	55.08^{ab}	39.62^{b}	0.0875^{b}	54.31^{c}	68.16^{ab}	3.47^{a}
V-PH	56.02^{a}	40.46^{ab}	0.0887^{ab}	54.55^{bc}	66.43^{b}	3.49^{a}
SWE	51.1^{b}	36.14^{c}	0.0824^{c}	50.96^{d}	61.46^{c}	3.08^{b}
A-PH + SWE	56.25^{a}	40.99^{a}	0.0898^{a}	56^{a}	72.3^{a}	3.44^{a}
V-PH + SWE	56.7^{a}	41.14^{a}	0.0894^{a}	55.84^{ab}	71.23^{ab}	3.46^{a}
H_2O	36.68^{c}	25.72^{d}	0.0517^{d}	33.52^{e}	45^{c}	2.29^{c}
Interaction						
L1 × A-PH	57.89	41.11	0.0903^{b}	57.81	71.4	3.68
L1 × V-PH	58.63	42.11	0.0918^{ab}	57.28	69.43	3.66
L1 × SWE	53.7	38.1	0.0850^{c}	54.18	65.1	3.17
L1 × (A-PH + SWE)	58.4	42.53	0.0927^{a}	59.22	76.43	3.63
L1 × (V-PH + SWE)	59	42.92	0.0920^{a}	59.18	74.63	3.64
L1 × H_2O	38.54	26.86	0.0593^{d}	35.65	50.33	2.28
L2 × A-PH	52.28	38.14	0.0848^{a}	50.81	64.93	3.28
L2 × V-PH	53.41	38.82	0.0857^{a}	51.83	63.43	3.33
L2 × SWE	48.49	34.19	0.0798^{b}	47.74	57.83	2.98
L2 × (A-PH + SWE)	54.1	39.45	0.0870^{a}	52.81	68.16	3.27
L2 × (V-PH + SWE)	54.11	39.35	0.0863^{a}	52.51	67.83	3.34
L2 × H_2O	34.82	24.58	0.0440^{c}	31.39	39.8	2.3

Light intensities: L1 (100% light intensity), L2 (50% light intensity), different biostimulants: animal protein hydrolysate (A-PH), vegetal protein hydrolysate (V-PH), seaweed extract (SWE), (A-PH + SWE) and (V-PH + SWE). Plants treated with H_2O served as the control. Different letters within each column indicate significant differences according to the least significant difference (LSD) ($P \leq 0.05$).

The interaction of light intensity and biostimulant application had a significant effect ($P \leq 0.01$) on the total anthocyanin content in violets (Table 2). The highest anthocyanin concentration (0.0927 µmol g^{-1} FW) under 100% light intensity was found in plants treated with A-PH + SWE, though no significant difference was observed between this treatment and V-PH + SWE. The lowest anthocyanin content (0.0593 µmol g^{-1} FW) was recorded in control plants. Under 50% light intensity, the maximum anthocyanin content (0.0870 µmol g^{-1} FW) was also found in plants treated with A-PH + SWE, while the lowest content (0.0440 µmol g^{-1} FW) was associated with control plants (Table 3).

Both light intensity and biostimulant application strongly influenced DPPH radical scavenging activity ($P \leq 0.01$), but their interaction did not have a significant effect (Table 2). DPPH activity increased by 12.66% under 100% light intensity compared to shaded conditions. The highest DPPH activity (56%) was observed in plants treated with A-PH + SWE, though no significant difference was found between A-PH + SWE and V-PH + SWE. The lowest DPPH activity (33.52%) was recorded in untreated plants (Table 3).

The simple effects of light intensity and biostimulant application had a significant impact on antioxidant activity as measured by the ABTS method ($P \leq 0.01$), while their interaction did not show a significant effect (Table 2). Antioxidant activity, as assessed by the ABTS method, increased by 12.51% under 100% light intensity compared to plants grown under 50% light intensity. The highest antioxidant activity (72.3%) was recorded in plants treated with A-PH + SWE, although this treatment did not differ significantly from the V-PH + SWE and A-PH treatments. The lowest antioxidant activity (45%) in the ABTS assay was found in untreated plants (Table 3).

Light intensity and its interaction with

biostimulant application did not significantly affect antioxidant activity as measured by the FRAP method. However, biostimulant application had a strongly significant impact ($P \leq 0.01$) on antioxidant activity in the FRAP assay (Table 2). The highest antioxidant activity by the FRAP method (3.49 mmol Fe^{+} g^{-1}) was observed in plants treated with V-PH, though no significant differences were found among the V-PH, A-PH, A-PH + SWE, and V-PH + SWE treatments. The lowest antioxidant activity (2.29 mmol Fe^{+} g^{-1}) by the FRAP assay was recorded in control plants (Table 3).

Discussion

The current study showed that light intensity significantly affected morphological traits in violets. Fresh and dry weights of the aerial parts increased under full sunlight. Variations in light intensity affected photosynthesis and cellular metabolism, influencing plant yield and growth parameters (Kaluzewicz et al., 2017). Our results indicated that while leaf area and leaf width decreased under full sunlight, leaf length increased with higher light intensity. Light intensity could modify leaf anatomical and morphological traits during developmental stages, resulting in alterations in the number and size of mesophyll cells (Wilson and Cooper, 1969). Leaf expansion was determined by both cell division and cell enlargement (Friend and Pomeroy, 1970). Shaded plants often exhibited cell elongation as a strategy to escape low light conditions, which enhanced light absorption and photosynthetic efficiency (De Oliveira et al., 2023). Plants adapted to varying light conditions by modifying morphological and physiological responses, such as increasing plant height and leaf area (Wang et al., 2021).

The interaction between light intensity and biostimulant application significantly influenced leaf area. Generally, green leaf area was closely linked with photosynthetically active radiation (PAR) interception and biomass accumulation. Under low light conditions, some plant species increased leaf area to enhance light absorption and improve photosynthetic efficiency (Cai et al., 2007). However, this often led to a decrease in leaf thickness and biomass yield per unit leaf area (Asaeda et al., 2005). Our findings aligned with those of Rezaei et al. (2018), who observed that leaf area increased with reduced light levels up to 50%. Hirano et al. (2019) found that total plant mass in *Datura inoxia* and *D. stramonium* decreased under lower light intensity, while total leaf area per plant increased. In our study, the application of biostimulants positively improved morphological traits compared to untreated plants. Specifically, the application of SWE and PHs enhanced morphological parameters relative to control plants. Biostimulants were known to promote plant growth and development by modifying cell division and enhancing water and nutrient uptake, leading to increased total dry biomass (Lima et al., 2019).

Extensive literature supported the positive effects of biostimulants on the morphological traits of various crops. For instance, Elansary et al. (2016) reported that SWE application via drench method improved the performance of Spiraea niponica and Pittosporum eugenioides under drought conditions by increasing leaf number, leaf area, dry weights, and some physiological responses. Similarly, Mafakheri and Asghari (2018) observed significant increases in shoot lengths, fresh weights, and dry weights in *Trigonella foenum-graecum* treated with SWE, compared to treatments with humic acid and chemical fertilizers. Consentino et al. (2020) found that V.PH-treated celery exhibited higher fresh weight compared to A.PH-treated plants. Overall, the combined application of PHs and SWE provided additive effects on the morphological parameters of violets compared to individual treatments, with PHs showing more pronounced effects.

Furthermore, increased light intensity led to significantly higher levels of total phenolic and flavonoid content compared to shaded plants. Bioactive compounds such as phenolics and flavonoids played crucial roles in defense mechanisms against biotic and abiotic stresses by mitigating oxidative damage through free radical chelation (Lattanzio, 2013; Kah-Yaw et al., 2019). The significance of phenolic compounds in human health was attributed to their anti-inflammatory and antioxidant properties, which could have preventive and therapeutic effects against various diseases (Biondi et al., 2021). Elevated levels of these compounds under higher irradiance had been reported in several studies, including Pan and Guo (2016), who demonstrated that different light intensities influenced the accumulation of flavonoid glycosides in *Epimedium pseudowushanense*.

In previous research by Muttaleb et al. (2018), the highest concentrations of total phenolics and flavonoids in *Piper betle* L. were observed under full sunlight. Additionally, it was noted that the application of biostimulants resulted in a significant increase in these compounds in violet plants compared to untreated controls. Biostimulants were known to activate secondary metabolism, leading to enhanced production of biochemical compounds, increased nutrient uptake, and improved photosynthetic efficiency

(Baltazar et al., 2023). These findings were linked to the biostimulants' effects on nutrient acquisition and the enhanced activity of key enzymes involved in the biosynthesis of bioactive compounds (Sun et al., 2024). Specifically, biostimulant applications strongly stimulated the phenylalanine ammonia-lyase (PAL) enzyme, resulting in higher accumulation of phenolics and flavonoids in plant tissues (Giordano et al., 2022; Zamljen et al., 2023).

Our findings aligned with previous research on the application of Protein Hydrolysates (PHs) and seaweed extracts (SWE). Rouphael et al. (2018) demonstrated a significant impact of PHs and seaweed extracts on total phenol content in greenhouse spinach. Additionally, Consentino et al. (2020) reported that applications of animal and plant-derived PHs enhanced total phenolic content in celery by 36.9% and 20.8%, respectively, compared to control plants. Similar increases in phenolic concentrations had been documented by Aremu et al. (2022) for *Abelmoschus esculentus* and by Giordano et al. (2022) for lettuce.

Anthocyanins, water-soluble compounds that inhibit reactive oxygen species (ROS) production in the photosynthetic electron transport system, were known to protect plants from high light stress by absorbing excessive light (Stetsenko et al., 2020; Zhao et al., 2022). Light had been identified as a crucial environmental factor influencing anthocyanin content in plants (Grisebach, 1982). In this study, plants grown under full irradiance exhibited higher anthocyanin levels compared to those in shaded conditions. Zhang et al. (2018) revealed that high light intensity significantly up-regulated the expression of genes involved in anthocyanin biosynthesis in red leaf lettuce. Moreover, biostimulant application had been shown to increase anthocyanin content. For example, Soppelsa et al. (2018) found that foliar application of alfalfa hydrolysate increased anthocyanin content in apples compared to control plants, while Szczepanek et al. (2020) reported that Kelpak seaweed extract positively influenced anthocyanin content in carrots both post-harvest and during storage.

Currently, there was a growing focus in the food industry and health research on substituting synthetic antioxidants with natural, plant-derived antioxidants (Manessis et al., 2020; Luo et al., 2022). The antioxidant activity of medicinal plants should have been assessed using multiple methods due to the diverse mechanisms of antioxidant action (Qasim et al., 2017). In this experiment, the antioxidant activities of violet extracts were evaluated using radical scavenging assays (DPPH and ABTS) and the ferric reducing antioxidant power (FRAP) assay. Phenolic compounds exhibited antioxidant activity through hydrogen atom transfer from hydroxyl groups and electron transfer followed by proton transfer (Csepregi et al., 2016). Flavonoids also played a critical role in antioxidant activity through their free radical scavenging ability (Ee et al., 2019).

Our results demonstrated that antioxidant activity was higher in violet plants exposed to full light compared to those in shaded conditions. However, no significant difference was observed in FRAP values between the two light conditions. Several studies had confirmed that light exposure enhanced antioxidant activity in medicinal herbs by upregulating genes involved in the metabolic biosynthesis of phytochemicals. Photoreceptors activated signaling pathways upon photon absorption, leading to changes in gene expression and alterations in phytochemical profiles and antioxidant potential (Folta and Carvalho, 2015). Karimi et al. (2013) showed that DPPH and FRAP assays, along with phenolic and flavonoid compounds, were enhanced in all three varieties of *Labisia pumila* Benth under high light intensity. Similarly, our experiment found that all investigated biostimulants significantly improved antioxidant activities in violet extracts for DPPH, ABTS, and FRAP assays.

A substantial body of literature had examined the antioxidant activity of various crops treated with biostimulants. Mannino et al. (2020) reported a 38% increase in ABTS and an 11% increase in DPPH radical scavenging activity in tomato fruit treated with seaweed and yeast extracts. Additionally, Cristofano et al. (2023) observed increased DPPH, ABTS, and FRAP values in lettuce treated with PHs compared to untreated plants. Overall, this study indicated that increased total phenolic, flavonoid, and anthocyanin contents were associated with higher antioxidant activities, except for FRAP values, in plants subjected to 100% light intensity. Furthermore, biostimulant applications led to substantial increases in all antioxidant activities, as well as total phenolic and flavonoid contents.

Conclusions

The current findings indicated that plants grown under 100% light intensity exhibited the highest concentrations of phenolic compounds, flavonoids, anthocyanins, and antioxidant activities compared to those grown under shaded conditions. Protein Hydrolysates demonstrated a more pronounced effect on the morphological and phytochemical traits in violets than seaweed

extract. Overall, the combined application of Protein Hydrolysates and seaweed extract, due to their synergistic effects, had a significantly greater impact on the evaluated traits of violets than the application of either component individually. Therefore, optimizing cultivation techniques using eco-friendly approaches could enhance crop yield and phytochemical contents in violets, especially in the absence of conventional fertilizers. Further research is recommended to explore these effects on other medicinal plants.

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Conflict of Interest
The authors indicate no conflict of interest in this work.

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