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RESEARCH ARTICLE

Effect of salt stress on the morphology, physiology and biochemical parameters of *Withania somnifera* (L.) Dunal: A medicinally important plant

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Abstract

Salt stress negatively impacts the growth of medicinal plants like *Withania somnifera* (L.) Dunal, resulting in loss of biomass and usability of its therapeutic secondary metabolic compounds, such as withanolides. This study investigates the effects of varying salinity levels (0 mM [control], 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 175 mM and 200 mM) on the morphological, growth and physiological parameters of *W. somnifera*. Morphological traits such as shoot length, root length and fresh and dry weight of the plants showed a negative correlation with salt stress. The results of biochemical analysis indicate a noticeable decline in chlorophyll a, b and total chlorophyll content, carbohydrate and protein levels with increasing salt concentration, while important phytochemicals like proline, phenols and flavonoid content exhibited an increase, consequently increasing the antioxidant activity executed by such metabolites as well. Most notably, Withaferin A and Withanolide A, important secondary metabolites responsible for the therapeutic properties of the plant, showed conflicting trends, with Withanolide A decreasing but Withaferin A increasing with an increase in stress, suggesting differences in the activities of their metabolic pathways under stress. The findings highlight the significant impact of salinity on both the growth and medicinal quality of *W. somnifera*, with the increase in Withaferin A with stress indicating an opportunity to explore this trend for achieving higher yield for commercial use in the pharmaceutical industry.

Keywords: abiotic stress; antioxidant activity; medicinal plants; salt stress; withanolides

Introduction

Salt stress is a major abiotic factor that limits plant growth and productivity worldwide. High soil or water salinity causes osmotic imbalance, ion toxicity and oxidative damage, leading to impaired photosynthesis, nutrient deficiency and stunted growth (1, 2). Typical stress symptoms include chlorosis, necrosis and reduced leaf area, reflecting reduced photosynthetic efficiency (3). Excess sodium (Na^+) and chloride (Cl^-) disrupt cellular homeostasis, damaging membranes and enzymes (4, 5). Osmotic stress restricts water uptake and cell expansion, while oxidative stress results from overproduction of Reactive Oxygen Species (ROS), which damage lipids, proteins and DNA (5). Salinity can reduce leaf area by up to 49 %, indicating severe metabolic impairment (6). Ion toxicity also interferes with the uptake of potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}), further disturbing metabolic balance (4). These physiological disruptions highlight the importance of developing salt-tolerant plant varieties for sustainable agriculture.

Medicinal plants are integral to traditional and modern medicine due to their diverse bioactive compounds. Among these, *Withania somnifera* (L.) Dunal, commonly known as *Ashwagandha* or Indian ginseng, stands out due to its broad spectrum of therapeutic properties, including anti-inflammatory, anti-stress, antioxidant and immunomodulatory effects (7, 8). This perennial shrub, belonging to the Solanaceae family, is widely used in Ayurvedic medicine and is increasingly recognised in modern pharmacology for its adaptogenic properties, which help the body resist physiological and psychological stress (9). The root of *W. somnifera* is particularly valued for its rich content of withanolides, a group of steroidal lactones that contribute to its medicinal efficacy. These compounds have been shown to possess anti-cancer, anti-inflammatory, anti-leishmaniasis and neuroprotective properties, making *W. somnifera* a valuable plant in the pharmacological industry (10). Owing to its commercial value, *W. somnifera* cultivation has expanded beyond traditional growing regions, making it increasingly important to understand environmental factors that influence its yield and medicinal quality.

Like other medicinal plants, *W. somnifera* is sensitive to salinity, which adversely affects both growth and secondary metabolite production. Salt accumulation in soil reduces water and nutrient availability, impairing physiological performance. Maintaining withanolide biosynthesis under stress is essential to preserve its therapeutic efficacy (11). Any reduction in withanolide content not only lowers pharmaceutical quality but also diminishes the plants' market value, directly impacting the livelihoods of growers and the supply chain of herbal industries. Despite its economic and medicinal importance, studies on *W. somnifera* responses to salinity remain limited, hindering its sustainable cultivation in salt-affected regions.

Plants exhibit multiple adaptive mechanisms under salinity, such as osmotic adjustment, ion compartmentalisation and antioxidant defence activation (12–14). For medicinal plants, maintaining secondary metabolite production is especially important since these compounds are influenced by environmental factors (15). A decline in such metabolites directly affects therapeutic efficacy. Previous research has mainly focused on food crops such as wheat and barley, whereas medicinal species like *W. somnifera* have received limited attention despite their pharmaceutical potential and role in agroecological diversification (16–19). Understanding its adaptive and metabolic responses to salinity could thus support both environmental resilience and the global herbal drug industry.

A few studies that do exist on medicinal plants under salt stress have shown that secondary metabolite production can be significantly affected. For example, research on *Mentha piperita* revealed the MAPK-mediated regulation of essential oil production and how it fares under NaCl stress (20). Similarly, in *Ocimum basilicum* L., salinity affected growth parameters quite considerably, but some cultivars were found to be better at tolerating salinity stress than others (21). However, most research addresses short-term responses, overlooking prolonged salinity exposure. The influence of salt stress on secondary metabolism remains poorly understood. Understanding these effects is essential for sustaining the therapeutic potential of medicinal plants under adverse conditions. For *W. somnifera*, understanding how salt stress affects the biosynthesis of withanolides and other secondary metabolites is vital for maintaining its therapeutic potential under adverse conditions.

This study investigates the effects of varying NaCl concentrations on the morphology, physiology and biochemical composition of *W. somnifera* to address existing research gaps. The findings aim to elucidate the plants' adaptive responses to salinity and their implications for medicinal compound production, contributing to improved strategies for sustainable cultivation in saline environments.

Materials and Methods

Plant material

Authenticated seeds of *W. somnifera* (Arka ashwagandha variety) were procured from ICAR-Indian Institute of Horticultural Research, Hessaraghatta, Bengaluru, India (13°08'04.7" N 77° 29'27.2" E). The collected seeds were surface sterilised with a soap solution for 1–2 min and then sodium hypochlorite (NaOCl) for 30 sec. A final rinse with running distilled water was given to wash off any leftover soap and NaOCl. For an initial viability study, seed trays were prepared with cocopeat and the seeds were allowed to germinate for 10 days. The cocopeat was autoclaved to achieve sterility and remove any chance of foreign contamination. When the seeds were confirmed to be viable, a proper pot experiment was set up with 9 different sets.

Experimental design

In March 2024, in the Polyhouse at CHRIST (Deemed to be University), Central Campus, Bengaluru, 9 sets of *W. somnifera* were labelled control, 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 175 mM and 200 mM, indicating the concentration of NaCl stress to be administered to the sets. These were set up following a randomised complete block design (RCBD). For each set, 3 pots representing 3 triplicates were set up, totalling 27 pots for the entire experiment. Each of the pots had 1.5 kg of soil and a uniform weight of 2 g, corresponding to approximately 200 counts of *W. somnifera* seeds, was sown in each pot. Salinity stress in each set was administered by direct application of different concentrations of NaCl solution in double-distilled water into the soil, starting from day 10, at an interval of every 5 days, till day 40. Pots were irrigated with normal tap water (non-saline) 3 days after administration of each saline water treatment. The plant biomass was finally harvested on the 45th day.

Measurement of vegetative growth characteristics

To evaluate the growth and development of 45-day-old germinated seedlings, the length of the shoot, root, number of leaves on each sapling and fresh and dry weight of each sapling were considered. After harvest, the plants were gathered and cleaned under running tap water to get rid of any dirt or debris from the roots. For both the control and test concentrations, the average length of shoot and root, the average number of leaves on a single plant and the fresh biomass of the plant were considered. To measure the lengths of the shoots and roots, a string was used to guide along the length of the shoot and root and then the string was measured against a standard measuring scale. For fresh biomass, a calibrated analytical weighing balance was used (Aczet Pvt. Ltd., Bengaluru, India).

Evaluation of biochemical parameters

Various biochemical parameters of both the control and experimental sets were evaluated, like total chlorophyll content (chlorophyll a, chlorophyll b and total chlorophyll), total carbohydrate content, total protein content, total proline content and antioxidant activity (both enzymatic and non-enzymatic activity).

Total chlorophyll content

The chlorophyll a and b and total chlorophyll levels were measured using a standard 80 % acetone extraction method (22). 0.1 g of Fresh Weight (FW) of leaf sample was taken in 5 mL 80 % acetone, refrigerated overnight and then homogenised and centrifuged at 5000 rpm for 5 min. The supernatant was taken and the optical densities were measured at 645 and 663 nm wavelengths using a spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The concentration of chlorophyll (a, b, total) was expressed as mg g^{-1} FW. Determination of chlorophyll a, b and total was carried out using the following formulas :

$$\text{Chlorophyll a (mg g}^{-1}\text{FW)} = (12.7 \times A_{663} - 2.69 \times A_{645}) \times V/1000 \times w \quad \text{(Eqn. 1)}$$

$$\text{Chlorophyll b (mg g}^{-1}\text{FW)} = (22.9 \times A_{645} - 4.68 \times A_{663}) \times V/1000 \times w \quad \text{(Eqn. 2)}$$

$$\text{Total chlorophyll (mg g}^{-1}\text{FW)} = (20.2 \times A_{645} + 8.02 \times A_{663}) \times V/1000 \times w \quad \text{(Eqn. 3)}$$

Where, A_{645} = absorption value at 645 nm, A_{663}= absorption value at 663 nm, V = total volume of filtrate in mL, w = fresh tissue weight in g

Total carbohydrate content

Carbohydrate estimation was carried out using the method followed, namely the phenol-sulphuric acid method (23). A 0.1 g sample of fresh leaf was homogenised in 5 mL of 2.5 N HCl, placed

in a boiling water bath for 3 hr and then cooled. The cooled crude homogenate was neutralised using sodium bicarbonate and centrifuged at 1000 g for 10 min. The supernatant thus obtained was taken (0.2 mL) and added to 1 mL phenol [2 % (v/v)] and 5 mL of sulphuric acid (H_2SO_4) [96 % (v/v)], while subject to a cold-water bath. The absorbance was then measured at 490 nm using a spectrophotometer. D-glucose anhydrous (dextrose anhydrous) was used to generate a standard graph, which gave the equation:

$$y = 0.0942x - 0.0064, R^2 = 0.9972 \quad \text{(Eqn. 4)}$$

Total protein content

The protein estimation assay used the standard Folin-phenol Lowrys' method (24). 0.2 mL of the sample extract made in protein buffer saline was taken and made up to 1 mL using distilled water and added to 5 mL of alkaline-$CuSO_4$ solution before incubation at room temperature for 10 min. Following this, 0.5 mL of Folin-Ciocalteu reagent was added to the mixture and further incubated at room temperature under dark conditions for 30 min. After this, absorbance was recorded at 660 nm using a spectrophotometer. Bovine serum albumin (BSA) was used as a standard to create a reference graph, with the final equation being:

$$y = 0.3995x + 0.0107, R^2 = 0.9971 \quad \text{(Eqn. 5)}$$

Total proline content

With modifications, the acid-ninhydrin method was utilised to measure overall proline content in the plant samples (25). 0.1 g of leaves was homogenised with 5 mL of 3 % sulfosalicylic acid and centrifuged. To 2 mL of the supernatant, 2 mL of 1.25 % ninhydrin reagent in glacial acetic acid was added. The mixture was heated in a boiling water bath for an hour and then cooled before measuring the absorbance at 508 nm using a spectrophotometer. L-proline was used as a reference standard to generate a graph, with the equation:

$$y = 1.075x + 0.017, R^2 = 0.9952 \quad \text{(Eqn. 6)}$$

Total phenolic content

The total phenol content was measured by the Folin-Ciocalteu assay (26). In this method, 25 µL of methanolic extract was mixed with 25 µL of Folin-Ciocalteu reagent (diluted with distilled H_2O in a 1:1 ratio). The mixture was allowed to stand for 2 min and then 75 µL of sodium carbonate (Na_2CO_3) solution (100 g/L) was added to the mixture and shaken, before being left to incubate under dark conditions at room temperature for 120 min. Following the incubation period, the absorbance was measured at 765 nm using a spectrophotometer. Gallic acid was used as a standard for creating a calibration curve, which gave the equation:

$$y = 0.02682x - 0.0056, R^2 = 0.9964 \quad \text{(Eqn. 7)}$$

Total flavonoid content

The total flavonoid content was measured using the modified Aluminium chloride ($AlCl_3$) colourimetric method (26). In this method, 50 µL of methanolic extract was mixed with 150 µL of 80 % methanol, 10 µL $AlCl_3$ [10 % (w/v)] and 10 µL 1M sodium acetate (CH_3COONa) and incubated at room temperature for 45 min. Then, the absorbance was recorded at 415 nm using a spectrophotometer. Quercetin was used as a standard and the calibration curve using the same yielded the equation:

$$y = 0.0108x - 0.0333, R^2 = 0.9977 \quad \text{(Eqn. 8)}$$

Antioxidant activity

Catalase activity: The catalase enzyme activity was analysed by the method outlined, with necessary modifications (27). First, an enzyme extract was made by dissolving 1 g of fresh sample in a 10 mL ice-cold 50 mM phosphate buffer (pH 7), centrifuging at 10000 rpm for 10 min at 4 °C. The supernatant was taken and a reaction mixture of 1.5 mL phosphate buffer, 1.2 mL H_2O_2 and 50 µL of the enzyme extract was prepared and the decrease in absorbance at 240 nm was noted at 0 min and 3 min intervals. The catalase activity was calculated with the following formula:

$$U\ mg^{-1} = (A_0\text{-}A_{180}) \times V_t/\varepsilon_{240} \times d \times V_s \times C_t \times 0.001 \quad \text{(Eqn. 9)}$$

where, $(A_0\text{-}A_{180})$ is the difference between the initial and final absorbance, V_t is the total volume of the reaction (3 mL) and ε_{240} is the molar extinction coefficient for H_2O, at $OD_{24}0$ (34.9 $mol^{-1}cm^{-1}$), d is the optical path length of the cuvette in cm (1 cm) V_s is the volume of the sample in mL (1 mL), C_t is the total protein concentration in the sample and 0.01 is absorbance change caused by 1 Unit of enzyme per min at 240 nm OD.

Ascorbate peroxidase enzyme activity

Ascorbate peroxidase (APX) activity was analysed using the method with necessary modifications (28). A crude enzyme extract was prepared by dissolving 0.5 g of fresh sample in an extraction buffer containing 50 mM phosphate buffer, pH 7.8, before centrifuging at 15000 g for 12 min at 4 °C. 0.1 mL of the supernatant thus obtained was mixed with 1.2 mL of 50 mM phosphate buffer (pH 7.8), 0.2 mL of 0.1 mM H_2O_2, 0.2 mL of 0.5 mM ascorbic acid and 0.2 mL of 0.1 mM EDTA. The decrease in absorbance was measured at 290 nm at 0 min and 5 min intervals. Based on the extinction coefficient, one unit of enzyme activity can be calculated as the amount of enzyme required to oxidise 1.0 µmol of ascorbate/min/mg protein.

$$\text{Unit activity (U/min/mg)} = \Delta A_{290} \times V_r/\varepsilon \times V_t \quad \text{(Eqn. 10)}$$

where, ΔA_{290} is the change in the absorbance/min, V_r is the total volume of reaction mixture in mL (1 mL), V_t is the volume of a sample taken in mL (0.1 mL) and ε is the extinction coefficient of ascorbate = 2.8 $mM^{-1}cm^{-1}$

Modified DPPH (2,2-diphenylpicrylhydrazyl) assay

The DPPH scavenging method was used after making slight modifications (29). An aliquot of 30 µL of the methanolic extract was taken and the volume was made up to 3 mL using methanol. To this mix, 1 mL of DPPH [0.004 % w/v] was added in dark conditions and the resultant mixture was incubated for 30 min in the dark. Then, the absorbance was measured at 513 nm using a UV-visible spectrophotometer. DPPH activity was expressed in percentages. Ascorbic acid in increasing concentrations was used as a reference for generating a standard graph, which gave the equation:

$$y = 0.0052x + 0.0164, R^2 = 0.9908 \quad \text{(Eqn. 11)}$$

Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power (FRAP) assay was performed by using the standard method (30). An aliquot of 1 mL of methanolic extract was mixed with 2.5 mL each of phosphate buffer (pH 7) and potassium ferricyanide ($K_3[Fe(CN)_6]$). The mixture was incubated at 50 °C for 20 min. 2.5 mL of trichloroacetic acid (10 %) was added to the mixture and centrifuged at 1000 rpm for 10 min. The upper layer of the solution (2.5 mL) was taken and then mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %). The absorbance was finally

measured at 700 nm and the results were expressed as µmol mL^{-1} Fe^{2+} equivalent.

Quantification of withanolide content through high-performance liquid chromatography

The withanolide content (particularly Withaferin A and Withanolide A content) of both the control and experimental plant sets was estimated using the method outlined in a previous study (31). Methanolic extract of the plants was filtered through a nylon microfilter (0.45 µM) and then subjected to HPLC analysis using Shimadzu LC 10A (Shimadzu Corporation, Kyoto, Japan) instrument equipped with Waters™ C-18, 25 0mm × 4.6 mm column (Waters Corporation, Bengaluru, India) and analysed using IRIS Spectral Processing software. Injection volume was set at 10 µL. The mobile phase used was a mixture of water and acetonitrile (HPLC grade) (30:70, v/v) at a flow rate of 1 mL min^{-1}. The column temperature was maintained at 40 °C and detection was carried out at 212 nm. The total run time was 8 min. Withanolide A and Withaferin A standards, at five different concentrations (0.2, 0.4, 0.6, 0.8 and 1 mg mL^{-1}) was employed to prepare a standard graph to help quantify the withanolides present in the sample. For Withaferin A, the standard curve R^2 value was obtained as 0.9354 and for Withanolide A, the R^2 was calculated to be 0.9235. The HPLC grade Withanolide A and Withaferin A (purity-99.3 %) were procured from Natural Remedies Pvt. Ltd., Bengaluru, India.

Statistical analysis

All of the above data were collected in triplicate and a statistical analysis of the readings was carried out using IBM SPSS Statistics for Windows, version 29.0.2.0 (IBM Corp., Armonk, N.Y., USA). One-way ANOVA and post-hoc Duncan's multiple range test (DMRT) were performed to determine any significant differences between control and treatment groups in the various chosen parameters. The findings were graphically represented as means ± standard error (SE), with alphabetical notation obtained from the groups assigned by DMRT.

Results and Discussion

Effect of NaCl stress on vegetative growth parameters

The present study provides insight into the morphological and physiological responses of *W. somnifera* under increasing NaCl stress. As shown in Fig. 1, shoot and root length exhibited a steady decline with increasing salt concentration, with the highest values recorded in the control (12 ± 0.97 cm) and a marked reduction at 200 mM NaCl. Interestingly, moderate concentrations (75–125 mM) induced slight variations, with root length peaking at 125 mM (6.8 ± 0.52 cm) before decreasing at higher salinity levels. Correspondingly, fresh and dry biomass also declined progressively, as demonstrated in Fig. 2, reflecting the cumulative inhibitory effect of salinity on water uptake, turgor maintenance and overall vegetative growth.

Abiotic stress factors like salinity are known to profoundly and negatively affect any plants' vegetative growth parameters (32). Similar growth reductions in *W. somnifera* indicate that although the species can tolerate mild salinity, higher concentrations lead to cellular dehydration and suppressed metabolic activity. The limited change in leaf number but visible reduction in leaf size suggests a regulatory adjustment rather than a complete morphological breakdown, possibly due to altered turgor pressure or hormonal imbalances affecting leaf expansion. Such changes may serve as early adaptive responses to mitigate water loss rather than indicators of irreversible damage.

Effect of NaCl stress on different biochemical parameters

For all three measurements of chlorophyll i.e., chlorophyll a, b and total chlorophyll, the highest values were recorded in the untreated control set and the lowest value was seen in the 200 mM NaCl treatment set, with a steady downward declining trend from the lowest to highest salinity (Fig. 3). Carbohydrate and protein levels also showed a similar declining trend from lowest (control) to highest (200 mM NaCl) stress, with significant differences between sets at $p \leq 0.05$ (Fig. 4). The observed decrease in chlorophyll content with increasing salinity is consistent with previous studies on various plants, where high salt levels led to chlorophyll degradation and impaired photosynthesis (1, 2). The significant reduction in carbohydrate and protein levels under salt stress aligns with findings

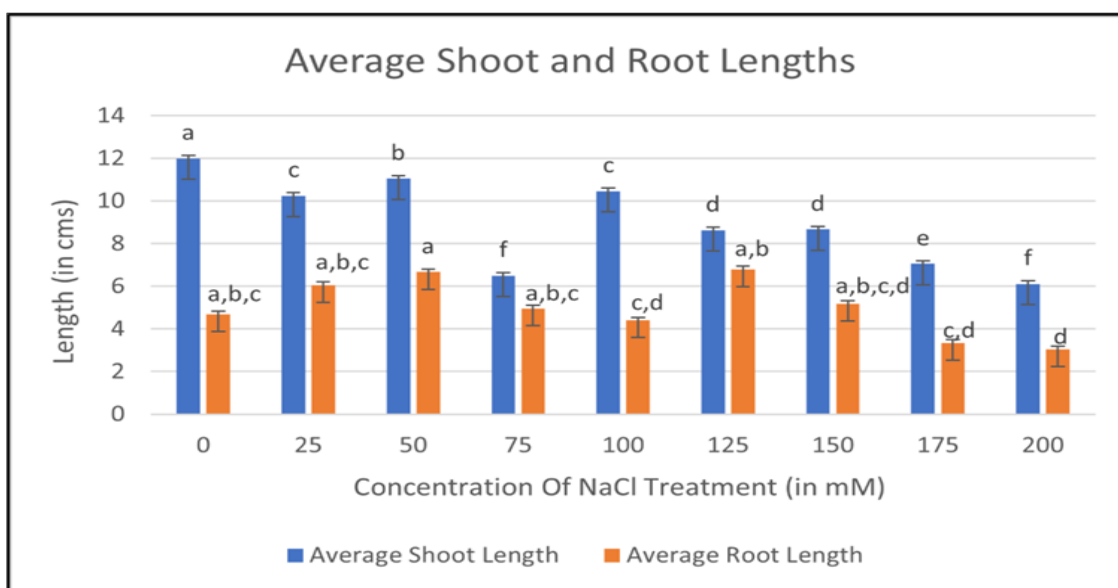

Fig. 1. Effect of NaCl stress on average shoot length and average root length of *Withania somnifera*.

Data represented as mean ± SE of triplicate readings. Means with common letters are not significantly different at $p \leq 0.05$, according to DMRT.

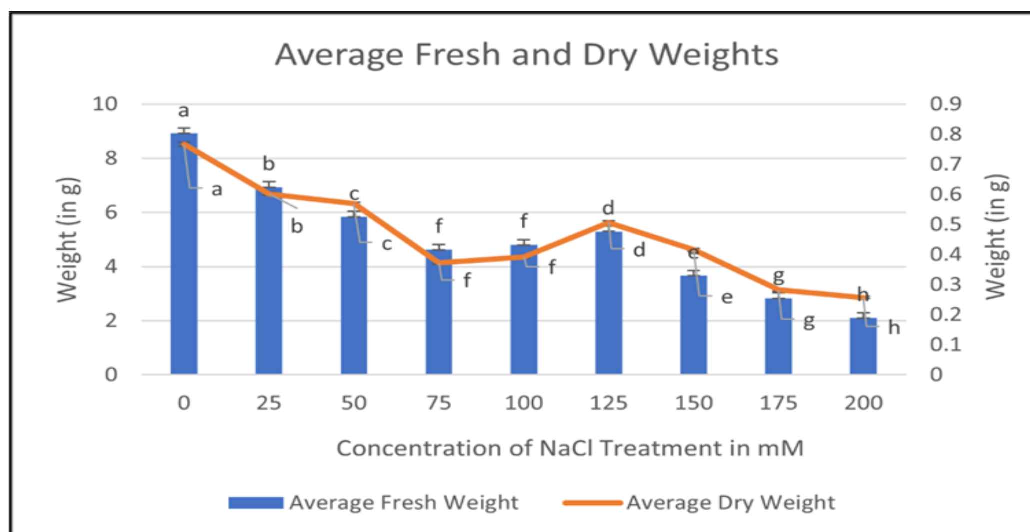

Fig. 2. Effect of NaCl stress on average fresh weight and dry weight of *Withania somnifera*.

Data represented as mean ± SE of triplicate readings. Means with common letters are not significantly different at $p \leq 0.05$, according to DMRT.

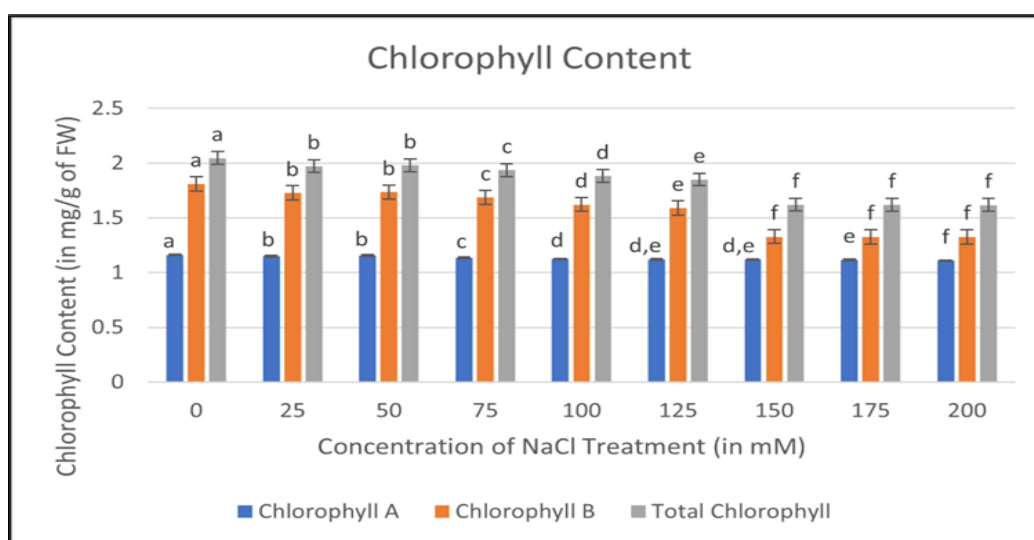

Fig. 3. Effect of NaCl stress on chlorophyll a, chlorophyll b and total chlorophyll content of *Withania somnifera*.

Data represented as mean ± SE of triplicate readings. Means with common letters are not significantly different at $p \leq 0.05$, according to DMRT.

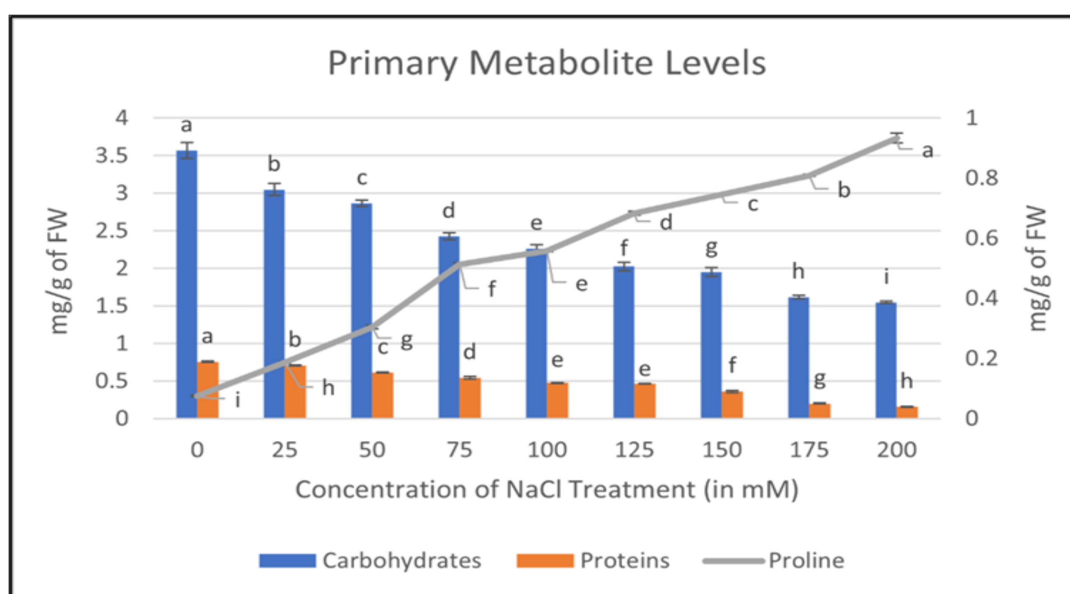

Fig. 4. Effect of NaCl stress on carbohydrate, protein and proline content of *Withania somnifera*.

Data represented as mean ± SE of triplicate readings. Means with common letters are not significantly different at $p \leq 0.05$, according to DMRT.

from studies on other medicinal plants, such as *Mentha piperita* and *Ocimum basilicum*, indicating a common physiological response to salinity (20, 21). However, in *W. somnifera*, the degree of reduction was notably sharper, suggesting increased sensitivity in chlorophyll stability.

Conversely, proline content, increased with increasing stress conditions, with the highest level of proline being observed in the 200 mM NaCl stress set (Fig. 4). Similarly, the secondary metabolites, namely phenolics and flavonoids, showed a steady increase with increasing NaCl stress, with the highest concentrations of both being observed in the 200 mM NaCl stress set (Fig. 5). This suggests an active metabolic reprogramming under stress. These compounds are known to participate in ROS scavenging, but in *W. somnifera*, the steep rise in phenolic and flavonoid content at 150–200 mM NaCl implies an enhanced antioxidant defence, likely linked to the regulation of phenylpropanoid pathways. This is supported by existing research that suggests that under salinity stress, plants accumulate compatible solutes like proline to combat the ion imbalance by decreasing the cytoplasmic osmotic potential and also to scavenge ROS (33, 34). In the context of ROS-scavenging, secondary metabolites like phenols and flavonoids play a significant role, as they have antioxidant properties. Phenolic compounds are known for their ability to act as hydrogen donors, which is a key mechanism in their antioxidant activity (35). Similarly, flavonoids, which are a subclass of phenolic compounds, also exhibit strong antioxidant properties (36). The observed accumulation of such compounds in this study aligns with the plants' need to maintain redox homeostasis under oxidative stress and also contributes to the enhancement of its medicinal potency.

Effect of NaCl stress on antioxidant activity

The enzymatic antioxidants, catalase (CAT) and APX showed increased activities with rising NaCl concentration (Fig. 6). This pattern complements the elevated non-enzymatic antioxidant capacity measured through DPPH and FRAP assays (Fig. 7). The parallel increase in phenolic and flavonoid levels with both DPPH and FRAP values indicates a strong correlation between secondary metabolite accumulation and enhanced antioxidant activity, as supported by existing literature (37). This relationship supports the

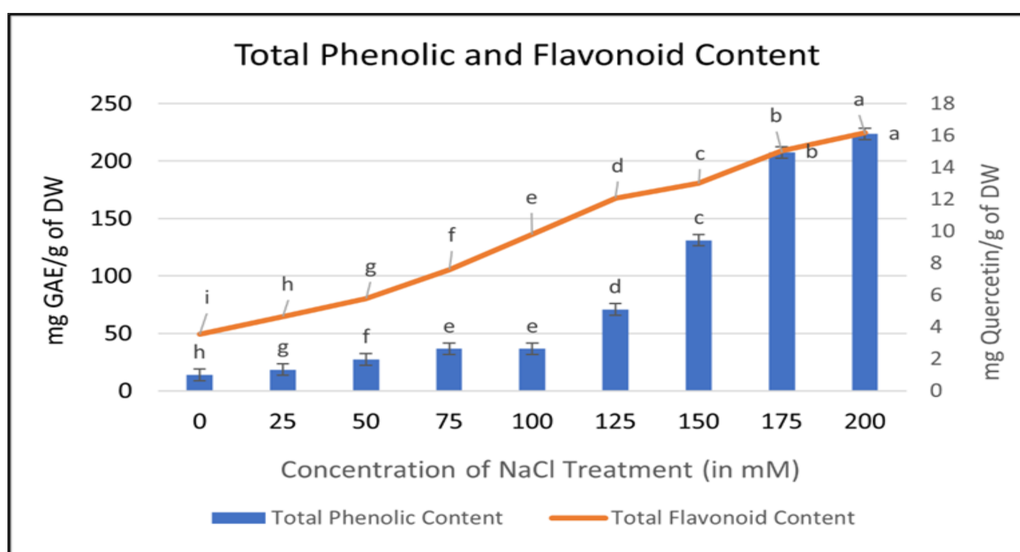

Fig. 5. Effect of NaCl stress on total phenolic and total flavonoid content of *Withania somnifera*.

Data represented as mean ± SE of triplicate readings. Means with common letters are not significantly different at $p \leq 0.05$, according to DMRT.

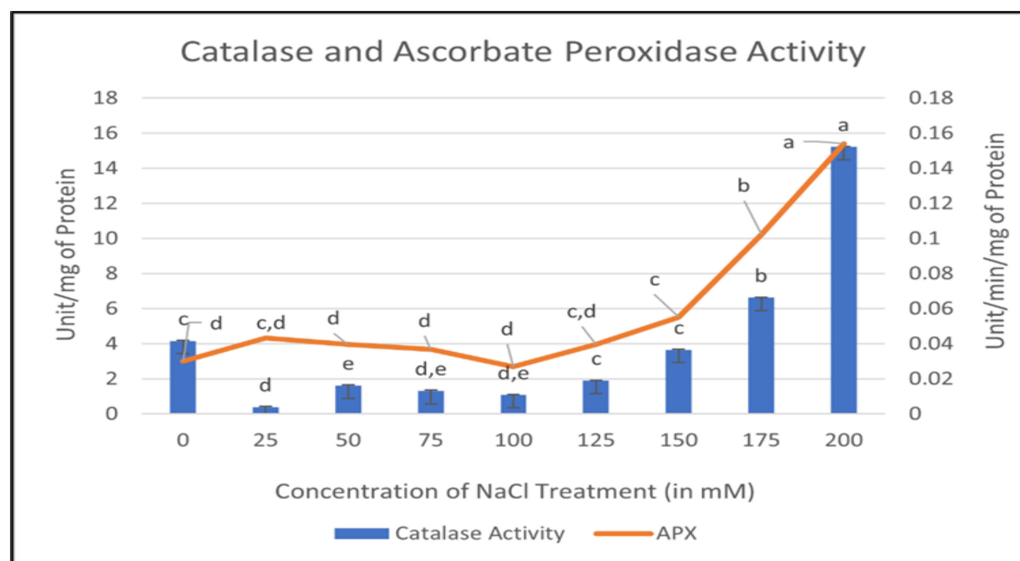

Fig. 6. Effect of NaCl stress on CAT and APX activity of *Withania somnifera*.

Data represented as mean ± SE of triplicate readings. Means with common letters are not significantly different at $p \leq 0.05$, according to DMRT.

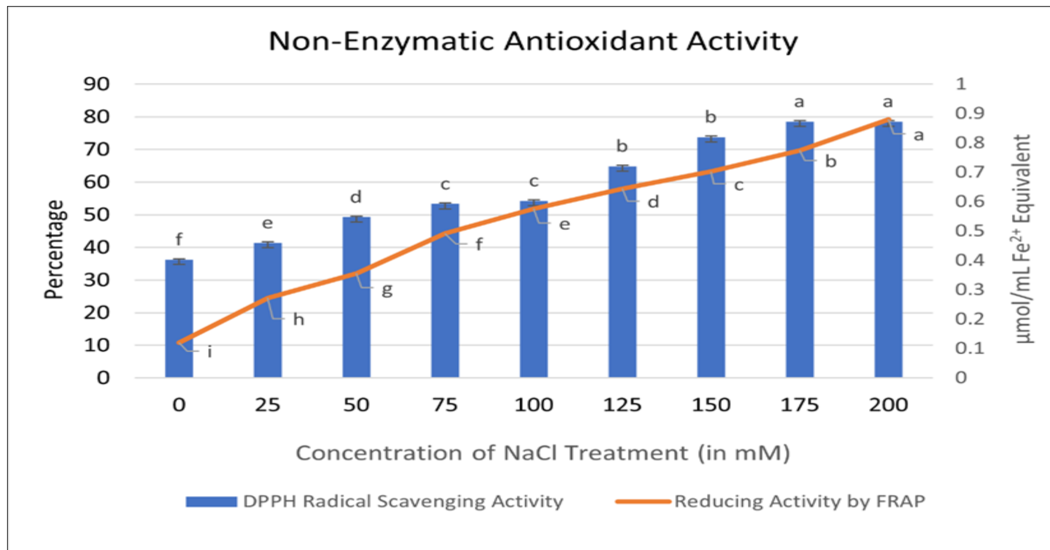

Fig. 7. Effect of NaCl stress on the non-enzymatic antioxidant activity of *Withania somnifera* as per DPPH and FRAP assays.

Data represented as mean ± SE of triplicate readings. Means with common letters are not significantly different at $p \leq 0.05$, according to DMRT.

hypothesis that *W. somnifera* adapts to salinity stress through the activation of its antioxidant machinery, where phenolics and flavonoids act synergistically with enzymes to neutralise ROS and stabilise cellular integrity. Compared to prior studies on other medicinal plants, the pronounced increase in antioxidant response observed here suggests that *W. somnifera* possesses a robust biochemical defence, potentially linked to the same biosynthetic machinery that governs withanolide production (38–41). This highlights a dual physiological advantage (oxidative protection and maintenance of therapeutic metabolites) under saline stress.

Effect of NaCl stress on withanolide levels

The quantification of Withaferin A and Withanolide A revealed divergent responses to salinity (Fig. 8). In this context, previous studies have shown that salinity stress initially increases secondary metabolite levels in *Solanaceae* plants, including Withaferin A in *W. somnifera*, as a defence response (42, 43). However, excessive stress shifts energy towards survival, reducing secondary metabolite accumulation (42–44). The same instance has been reported in both Withaferin A and Withanolide A levels in the current study, showcasing a sharp decline under heavy salt stress (beyond 125 mM). Both compounds increased up to 125 mM NaCl, beyond which Withanolide A declined while Withaferin A remained comparatively higher. For Withanolide A, although an increase with increasing levels of NaCl stress is observed, the highest Withanolide A levels are observed in the control set with no stress (Fig. 8). However, another important observation is that the levels of Withanolide A were highest in the control set, but Withaferin A levels increased with increasing salt stress. This suggests that Withaferin A and Withanolide A are responding differentially to stress. This can be explained by the fact that Withanolide B has been discovered to be an early intermediate in the withanolide biosynthetic pathway, branching into Withaferin A and Withanolide A. The cytochrome P^{450} enzyme WsCYP71B35 is involved in the Withanolide A pathway (44). It is plausible to hypothesise that NaCl stress may disrupt WsCYP71B35 activity, leading to reduced Withanolide A synthesis and increased conversion of Withanolide B into Withaferin A, explaining the observed metabolite shifts (44).

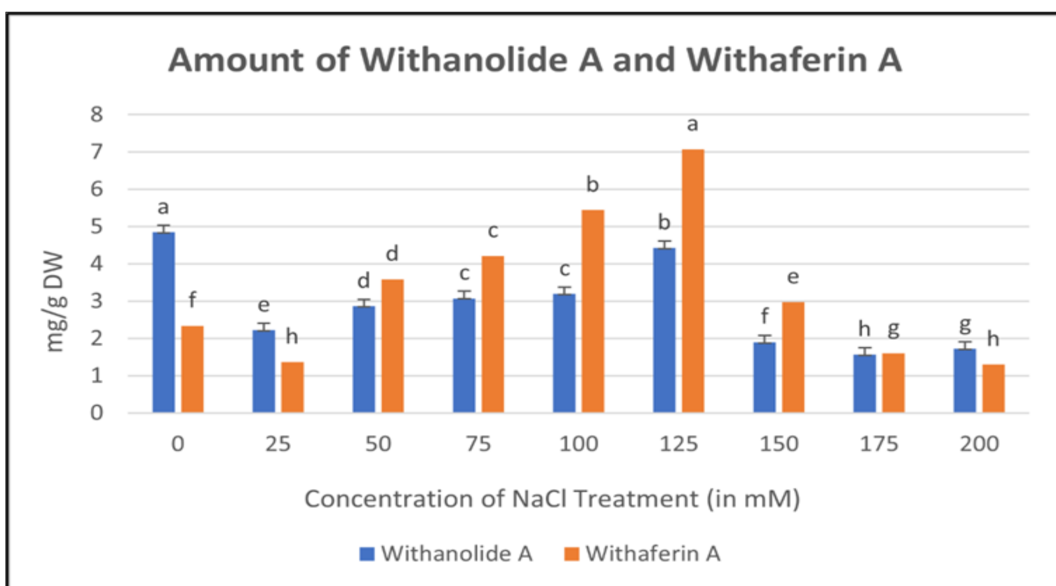

Fig. 8. Effect of NaCl stress on the Withanolide A and Withaferin A levels of *Withania somnifera* as per FRAP assay.

Data represented as mean ± SE of triplicate readings. Means with common letters are not significantly different at $p \leq 0.05$, according to DMRT.

Practical and long-term implications

The observed physiological and metabolic responses of *W. somnifera* under salinity highlight its capacity to tolerate moderate salt concentrations through biochemical adjustment rather than structural resilience. However, sustained exposure to high salinity (≥ 150 mM) leads to significant reductions in photosynthetic pigments and protein levels, which can compromise overall productivity. From an ecological perspective, understanding these adaptive limits is critical for expanding *W. somnifera* cultivation into semi-arid and salt-affected soils, particularly in regions where land degradation threatens traditional agriculture. Economically, these findings underscore the potential to breed or select *W. somnifera* cultivars with enhanced salt tolerance while maintaining high withanolide yield. Such efforts could reduce crop losses in saline-prone regions and stabilise raw material supply for the herbal pharmaceutical industry. Furthermore, integrating biostimulant-based strategies or moderate salinity induction could be explored as cultivation practices to enhance Withaferin A biosynthesis without compromising plant health.

Conclusion

The present study elucidates the detrimental effects of salt stress on the morphological, growth and physiological parameters of *W. somnifera*. Morphological traits, including shoot and root length and fresh and dry weight of the plants, were adversely affected, indicating overall stunted growth under high salinity conditions. Increasing salinity levels led to significant reductions in chlorophyll, carbohydrate and protein content, while proline, phenolics and flavonoid levels increased as an adaptive response to osmotic stress. This, in turn, increased the antioxidant activity of the plants, confirmed by both enzymatic and non-enzymatic tests, confirming the role of secondary metabolites as osmoprotectants and stress-response molecules. Most significantly, medically important secondary metabolites, namely Withaferin A and Withanolide A, showed differential response to salt stress, potentially revealing a method to boost withaferin production in *W. somnifer*a for therapeutic uses. These findings highlight the critical impact of salt stress on the growth and medicinal quality of *W. somnifera*, a plant renowned for its therapeutic compounds.

The observed physiological responses to salinity stress suggest a common pattern of stress adaptation seen in other medicinal plants as well. However, the reduction in key biochemical components essential for the plants' medicinal properties highlights the potential threat to the production and efficacy of *W. somnifera* in saline environments. Developing salt-tolerant cultivars through genetic and biotechnological interventions is imperative for sustaining their cultivation and ensuring their therapeutic value. Future research should aim to dissect the molecular mechanisms governing salt tolerance in *W. somnifera* and conduct long-term field studies to translate laboratory findings into practical cultivation strategies. By addressing these challenges, we can enhance the resilience of *W. somnifera* to salinity stress, thereby supporting its continued use in traditional and modern medicine.

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Authors' contributions

AS conceptualised the design of the study, carried out the biochemical analysis of the plant samples, performed the statistical analysis and drafted the manuscript. PN conceptualised the design of the study, critically evaluated and edited the manuscript for corrections. All the authors read and approved the final manuscript.

Compliance with ethical standards

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