Section A-Research paper
ISSN 2063-5346

SODIUM CHLORIDE EXPOSURE MODULATES PHYSIOLOGICAL, BIOCHEMICAL AND ARTEMISININ BIOSYNTHESIS IN ARTEMISIA ANNUA L.

Nafish Ahmad[1], Riyazuddeen Khan[1]*, Iffat Zareen Ahmad[2]
[1]Department of Environmental Science, Integral University, Dasauli Kursi Road, Lucknow, 226026
[2]Department of Bioengineering, Integral University, Dasauli Kursi Road, Lucknow, 226026
Email: rkhan@iul.ac.in*

Abstract:

Recently, the increase of the global population, the loss of arable land, soil erosion, and the conversion of agricultural land into urban areas due to uncontrolled development have all put pressure on crop productivity. Artemisia annua L., is an annual plant local to Asia and Eastern Europe, is the primary source of the antimalarial chemical compound artemisinin, a sesquiterpene alkaloid found in maximum amounts in the leaves. Artemisinin, a bioactive compound present in the leaves of Artemisia, is thought to be an effective drug for malaria treatment. Under the stress of NaCl (sodium chloride), a randomized-design pot experimentation was undertaken with A. annua species. Plants were subjected to increasing soil-applied NaCl concentrations. All plant measurements were conducted 90 days prior to flowering. According to the results, A. annua plant showed a significant to NaCl stress; nonetheless, the highest dose of NaCl concentration was the most damaging to A. annua plants. It dramatically decreased plant growth and leaf chlorophyll 90 days after planting (DAP). However, the intermediate treatment has greatly enhanced the pre-flowering physiological and biochemical activities of A. annua plants, demonstrating that NaCl mediates oxidative stress. Intriguingly, both artemisinin concentration and production enhance dramatically with increasing NaCl concentrations.

Keywords: Artemisia annua L., artemisinin, physiological, biochemical activity, NaCl stress

1. Introduction

Plants exposed to NaCl experience substantial stress that is responsible to inhibit plant development, causes physiological imbalance, and ultimately causes plant mortality. In reality, exposure to NaCl can initiate a chain of responses that restrict growth, alter the photosynthetic and respiratory systems, and stimulate secondary metabolism (Acosta-Motos *et al*., 2017). The presence of NaCl in the soil alters the natural functioning of plants, resulting in decreased agricultural output and reduced plant development. Various strategies, including hyperaccumulation, phytochelation, and activation of the antioxidant defence system, are employed by plants to combat the toxicity of NaCl (Ahmad *et al*., 2019). However, under an appropriate NaCl stress, studies were reported to increase the growth attributes and yield of the artemisinin. Furthermore, the toxicity induce by NaCl were also comprehensively studied to prevent the NaCl contamination in the food chain by investigating the NaCl absorption potential of the plant and capability to metabolise it (Alharbi *et al*., 2022).

Artemisinin and its derivatives that are isolated in higher concentration were also reported to inhibit cancer and tumour growth in addition to their efficacy in treating malaria (Ma *et al*., 2020). However, millions of the low-income people of the world do not have access to artemisinin-derived medications due to the insufficient yield i.e., less than 0.5% of artemisinin in naturally harvested *A. annua*. The genetic enhancement of naturally-derived artemisinin was investigated and however, the highest artemisinin yield attained to date is 2% from the dry leaf weight (Badshah *et al*., 2018). Nevertheless, the artemisinin can be produced by chemical synthesis, although the process is complex and not commercially viable due to the yield reduction. Since the *A. annua* plant is the sole viable reservoir of artemisinin, the enhancement of artemisinin biosynthesis in the plant by inducing a substantial level of biotic and abiotic stress make it achievable (Al-Khayri *et al*., 2022).

According to the World Health Organization (WHO), artemisinin-free combination therapy (ACT) is the significant approach for the drug-resistant malaria parasites treatment (van der Pluijm *et al*., 2021). To increase artemisinin production, various scientific approaches have been employed, including genetic engineering and

chemical synthesis of the genetic pathway genes associated with artemisinin biosynthesis in *A. annua*. However, little success has been achieved owing to the high cost of the process or the complicated nature of the expression and regulation of the genes involved in the biosynthesis (Wani *et al*., 2021). Sensitivity of *A. annua* to abiotic stress-factors including UV, light, heavy metals, salinity and temperature has been reported to increase ROS production, which increases artemisinin yield by facilitating the fast transition of dihydroartemisinic acid to artemisinin although during biosynthetic pathway (Aftab *et al*., 2011). To boost the required output of artemisinin and utilise the land inappropriate for crop production (NaCl-affected land), it is necessary to deploy NaCl-tolerant cultivars in addition to scientific procedures that are less expensive and more practical.

The objective of the current experiment was to investigate the effects of NaCl stress on the on the growth attributes of the *A. annua* along with the alterations on its biochemical parameters like chlorophyll content, total sugar content, protein concentration, antioxidant activity (catalase) and artemisinin in *A. annua* cultivars with varying NaCl treatment concentration.

2. Methodology

The experimental procedure commenced with the surface sterilisation of the seeds of *A. annua* L. with a 0.02% solution of $HgCl_2$ for a period of 5 minutes with consistent agitation. Following the initial step, the seeds underwent a thorough rinsing process using de-ionised water. The treated seeds were then sown in seed beds with dimensions of 1 by 1 metre. After a period of one month from the initial sowing, seedlings of uniform size were carefully transplanted into clay pots, ensuring that only one seedling was placed in each pot. A limited quantity of water was administered subsequent to the transplantation procedure. Prior to the commencement of the transplantation procedure, it was ensured that each individual pot, with dimensions measuring 25 cm in diameter and 25 cm in height, was adequately prepared. This involved the meticulous filling of each pot with a carefully formulated mixture consisting of soil and farmyard manure (about 5 kg) in a precise ratio of 4:1. In advance of sowing, a standardized basal dose of potassium (K), phosphorus (P), and nitrogen (N) was administered uniformly to the soil, which had physio-chemical properties of sandy loamy texture with a soil-water ratio of about 1:2 and a pH of 7.5. The experimental design consisted of three replicates for each treatment, with each replicate comprising three individual plants. Consequently, the experimental design encompassed a total of nine pots per treatment, with each individual pot housing a solitary, thriving plant. The pots were adequately irrigated in accordance with the necessary water requirements. Different concentration of NaCl (0.05 mM, 0.1 mM, 0.5 mM, 1.0 mM and 2 mM) along with the control was applied (Naeem *et al*., 2019).

2.1. Plant growth attributes investigation

The plants from each treatment were meticulously uprooted, and subsequently, the fresh and dry weights of both leaves and stems were measured for each treatment in triplicate. The plant specimens were subjected to a desiccation process in a controlled environment. Specifically, they were placed in a hot-air oven that was carefully regulated to maintain a temperature of 80°C. This desiccation process lasted for 48 hours to measure the dry weight of both the leaves and stems of the plants.

2.2. Biochemical Parameter

2.2.1. Total contents of chlorophyll

The quantification of chlorophyll in the leaves was carried out employing the Lichtenthaler and Buschmann method (Ashenafi *et al*., 2023). The experimental procedure involved the utilization of fresh tissue samples obtained from the interveinal regions of leaves. These tissue samples were subjected to grinding using a pestle and mortar, employing 100% acetone as the solvent. The total content of chlorophyll was determined by recording the absorbance of the pigment containing solution at a wavelength of 680 nm using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan). The concentration was expressed as µg/ml relative to the fresh weight of the leaf (FW).

2.2.2. Estimation of protein

The quantification of proteins was conducted using the Folin-Ciocalteu reagent (Lowry *et al*., 1951). Bovine serum albumin (BSA), obtained from Himedia, was used as the standard for this analysis. A volume of 0.2 ml of either the sample or standard was introduced into a solution containing 0.5 ml of a complex-forming reagent that had been freshly prepared. The solutions were permitted to equilibrate at ambient temperature for 10 minutes. Subsequently, 0.1 ml of diluted Folin reagent was introduced into each respective test tube. The solution was then thoroughly mixed using a vortex mixer, ensuring proper homogenization. Following this

step, the mixtures were left undisturbed at ambient temperature for 30 minutes. The absorbance measurement was conducted at a wavelength of 680 nm following a 30-minute incubation period. The determination of protein concentrations in various samples was accomplished through the construction of a calibration curve.

2.2.3. Total aldoses

The total soluble sugars in the leaves were determined by adopting the protocol established by Prud Homme *et al*. (1992) with anthrone reagent. The samples were extracted from the plant material using an 80% ethanol solution. Plant materials were finely ground, heated at 60 ℃ for 6 hours, and filtered to obtain the sample extract that was used for the estimation of total soluble sugars. Subsequently, a volume of 6 ml of a recently prepared anthrone reagent, containing 150 mg of anthrone dissolved in 72% H_2SO_4, was carefully added to the test tubes along with the sample. The resulting mixture was heated and then cooled for 10 minutes each. The test tubes underwent incubation for a duration of 20 minutes under ambient conditions, specifically at room temperature ranging from 25 to 28 ℃. The optical density measurement was conducted using a UV-visible spectrophotometer (Shimadzu UV-1700, Tokyo, Japan) at 625 nm. The standard used in this experiment was glucose.

2.2.4. Determination of total ketoses

Resorcinol reagent was used to determine total reducing sugar for different treated samples (Roe, 1934). 100 µl of plant extract along with 1000 µl of resorcinol reagent (which consists of 100 mg of resorcinol and 250 mg of thiourea dissolved in glacial acetic acid (99.65 ml)), 1.9 ml of water and 7 ml of an 84% HCl solution were added in a test tube. The tubes were overtaxed to mix them, and they were then submerged in an 80°C water bath for ten minutes. Total fructose was calculated using the optical density at 520 nm after the tubes had been cooled. Fructose was used as the standard.

2.2.5. Starch quantification

The estimation of total starch content was conducted using the Anthrone method (Hodge & Hofreiter, 1962). In order to extract starch, a sample of well-grounded dry material of about 0.1 g was homogenized with 80% ethanol (5ml). The resulting mixture was then centrifuged at 5000 rpm for 20 minutes. The purpose of this centrifugation step was to effectively eliminate any remaining soluble sugars present in the sample. The pellet obtained in the experimental procedure involved the extraction of starch through the application of perchloric acid. The extraction involves the storage of pellets at a temperature of 4℃ for a duration of 20 minutes along with perchloric acid. The solution is then centrifuged to obtain a supernatant containing starch. The diluted extract is used for the determination of the starch content in the samples. In this experimental procedure, a total volume of 2 ml of starch extract was combined with 20% NaCl (5ml) solution and 2 ml of potassium iodide solution. Following the incubation period of 20 minutes, the mixture underwent centrifugation. After that, 5 mL of an alcoholic sodium hydroxide (NaOH) solution was added to the precipitate and then centrifuged. An additional 2 ml of a 0.25 N solution of alcoholic sodium hydroxide (NaOH) was introduced into the obtained supernatant. The mixture was allowed to stand until the blue coloration was no longer observable. Following the addition of 0.7 N HCl to the precipitate, the resultant mixture was subjected to heating for 2 hours. The mixtures underwent a dilution process, and the developed green colour was subjected to the addition of the anthrone reagent. Following the process of cooling the sample on an ice bath, the quantification of total starch content was done by recording the absorbance at 620 nm. The standard used in this study was glucose.

2.3. Antioxidant attributes

2.3.1. Rate of lipid peroxidation

The quantification of oxidative damage inflicted upon lipids present in leaves was conducted through the assessment of total 2-thiobarbituric acid reactive substances (TBARS), which were subsequently referred as equivalents to malondialdehyde (MDA). The content of TBARS in the extract was determined by employing the Cakmak and Horst methodology (Cakmak & Horst, 1991). The samples were prepared by grinding freshly chopped leaves (0.5 g) in 5 ml of 0.1% of TCA solution (trichloroacetic acid). The mixture was then centrifuged at 12000 rpm for 5 minutes to acquire a supernatant. The supernatant (1ml) was then mixed with 4 ml of the 0.5% solution containing thiobarbituric acid (TBA) dissolved in TCA (20%). Following this, the suspension was incubated for 30 minutes at 90℃. The reaction was then halted by lowering the temperature and placing it in a cold bath. The mixture was centrifuged, and the collected supernatant was quantified at 532 nm. The non-specific turbidity was accounted by eliminating the absorbance at 600 nm from the 532 nm. The TBARS content was quantified and reported in units of nmol g^{-1} of fresh weight.

2.3.2. Catalase activity

The evaluation of catalase activity was conducted in accordance with the methodology described by Chandlee & Scandalios, (1984), albeit with a slight adjustment. The extract containing the enzyme was centrifuged at 12500g for 20 minutes to acquire the supernatant (enzyme extract). The experimental setup consisted of an enzyme mixture consisting of 40µl of an extract containing enzyme, 50 mM potassium phosphate buffer solution having a pH of 7.0 (2.6 ml) and 15 mM H_2O_2 solution (0.4 ml). The process of H_2O_2 degradation was recorded by observing the gradual reduction in absorbance at a wavelength of 240 nm. The quantification of the enzyme's activity was presented as units per mg of protein, with each unit corresponding to the reduction of 1 mM of H_2O_2 per minute per mg of protein.

2.4. Artemisinin content using High Performance Liquid Chromatography (HPLC)

The present study aims to investigate the artemisinin content using the technique of HPLC. The estimation of artemisinin was conducted using dried leaf material weighing 1 gram. In order to facilitate the analysis, the pure artemisinin was used as a reference compound. The extraction process involved using a shaker set at a constant speed of 70 rpm for 24 hours with 20 ml of petroleum ether. Following a 24-hour period, the solvent was carefully separated and subsequently added to petroleum ether and this process was repeated thrice. The fractions of petroleum ether were combined and subjected to concentration under reduced pressure. The resulting residues were then treated with 10 ml of CH_3CN thrice for defatting purposes. The analysis and quantification of derivatized artemisinin were conducted using a reverse-phase column (C18 with a diameter of 4.6 mm). A premix of 10 mM K-phosphate buffer and methanol was employed as the mobile phase in a ratio of 40:60 and was delivered with constant flow rate (1ml/min) and injection volume 15µl. The absorbance of the analyte was detected at 254 nm and artemisinin was quantified using the below-mentioned formula:
(Area of sample/ Area of standard) X (Amount of standard/ Area of standard) X (Make up volume (mL)/ Weight of sample Injected vol (µL))

Results

2.5. Growth parameters

The observed impact of various concentrations of sodium chloride (NaCl) at levels of 0.05, 0.1, 0.5, 1, and 2 mM on the morphological traits of *Artemisia annua* was found to be statistically significant at 90 days after planting (DAP). The growth of *A. annua* plants was found to be significantly inhibited by the presence of high levels of NaCl in the soil. The most pronounced toxic effects were observed at concentrations of 2 mM of NaCl in the soil. Under conditions of elevated sodium chloride (NaCl) stress, the cultivar 'CIM-Arogya' exhibited a notable reduction in stem dry weight (SDW) and stem fresh weight (SFW) by 46.45% and 4.44% as compare to the control. Additionally, there was a rise in leaf dry weight (LFW), and leaf fresh weight more than 50% as compared to the control group that was treated with water (Figure 1). The results indicate that the application of NaCl at a concentrations upto 1mM led to an increase in both the dry and fresh weights of the plant's leaves compared to the control group representing the NaCl tolerance of *A. annua*.

Table 1. Impact of different concentration of NaCl on the growth attributes of *A. annua* after 90DAP.

Treatment of NaCl	Stem Fresh Weight (SFW)	Stem Dry Weight (SDW)	Leaf Fresh Weight (LFW)	Leaf Dry weight (LDW)
Control	75.67333 ± 1.46^{a}	43.73667 ± 2.03^{b}	254.1667 ± 31.9^{a}	123.0333 ± 2.25^{a}
0.05 mM	98.23 ± 2.78^{c}	51.63333 ± 2.54^{c}	1165.733 ± 2.24^{f}	277.37 ± 1.88^{e}
0.1 mM	127.93 ± 2.17^{d}	65.68333 ± 2.77^{d}	462.58 ± 2.63^{b}	192.0867 ± 1.12^{c}
0.5 mM	127.8067 ± 4.27^{d}	64 ± 2.50^{d}	667.5067 ± 2.22^{d}	196.42 ± 0.93^{d}
1 mM	84.13667 ± 4.91^{b}	41.16 ± 2.10^{b}	673.2233 ± 2.01^{e}	283.1467 ± 2.59^{f}
2 mM	72.30667 ± 1.4^{a}	23.4233 ± 4.41^{a}	502.8467 ± 2.49^{c}	$178.7533 \pm .2.23^{b}$

Data were represented as a mean ± SD of triplicates of each treatment are labeled with identical superscript alphabets are not significantly different (Duncan's Test, $p \leq 0.05$).

2.6. Biochemical attributes

NaCl treatment significantly enhanced the synthesis of photosynthetic pigment as compared to the control (Figure 1). The concentrations of 0.1 mM and 0.50 mM of NaCl treatment showed the presence of a significant amount of chlorophyll content in the *A. annua* plant. Similarly, the comparative study showed an increase in the production of total soluble protein under the treated conditions. As compared to the control, both chlorophyll and soluble proteins showed increased production after 90 DAP. However, the graph showed an increasing trend till 0.5 mM of NaCl treatment, which later showed a decline in production, representing the toxicity induced by the NaCl (Figure 1). At a concentration of 0.05 mM of NaCl, there was a 70.91% increase in the chlorophyll content, while at the above concentration, the production of chlorophyll increased by double as

compared to the standard. On the other hand, protein content showed a slight increase at a low concentration of about 9.13% and had the highest rise of 64.28% at 0.5 mM as compared to the control. However, the content of protein decreases at the highest dose of NaCl.

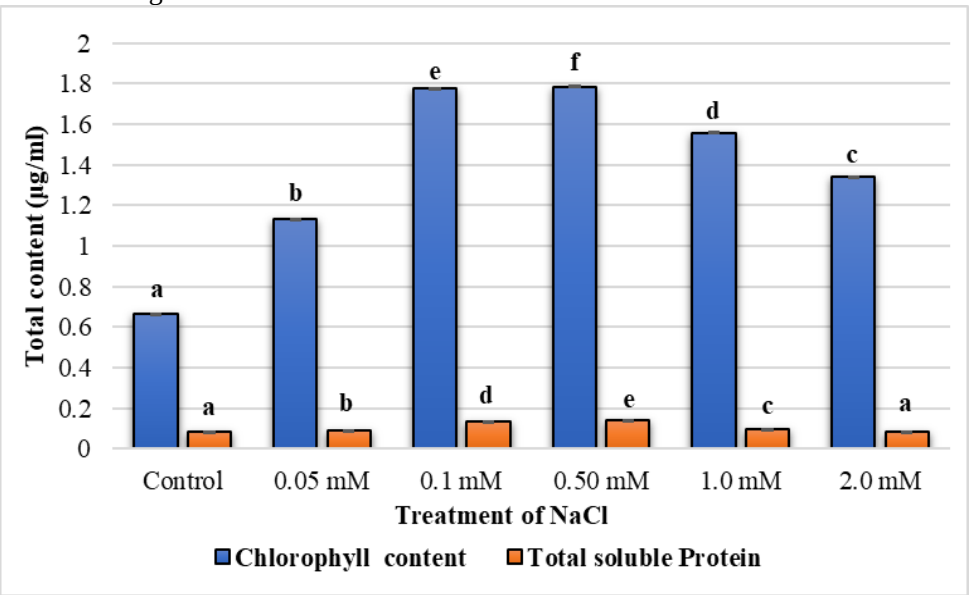

Figure 1. The quantity of total chlorophyll and the amount of soluble protein present in *A. annua* at 90 DAP are affected by varying NaCl concentrations. Data were represented as a mean ± SD of triplicates of each treatment are labeled with identical superscript alphabets are not significantly different (Duncan's Test, $p \leq 0.05$).

Furthermore, low NaCl levels (0.05 mM and 0.1 mM) showed no significant impact on the total aldose and starch content as compared to the untreated plant, which showed an approximately 4% and 10% rise in the content, respectively. However, a significant rise in the ketose sugar was observed at the initial concentration of the NaCl treatment (double), which declined as the toxic effect of the NaCl on the plant declined at 90 DAP as compared to the control. There is a reduction in ketose content of 83.61% at a dose of 2 mM, while an increase of about 97.98% was observed for aldose at the highest dose. A decline in aldose and ketose was also observed at the high dose of NaCl, but the concentration as compared to the control was still substantially high (Figure 2).

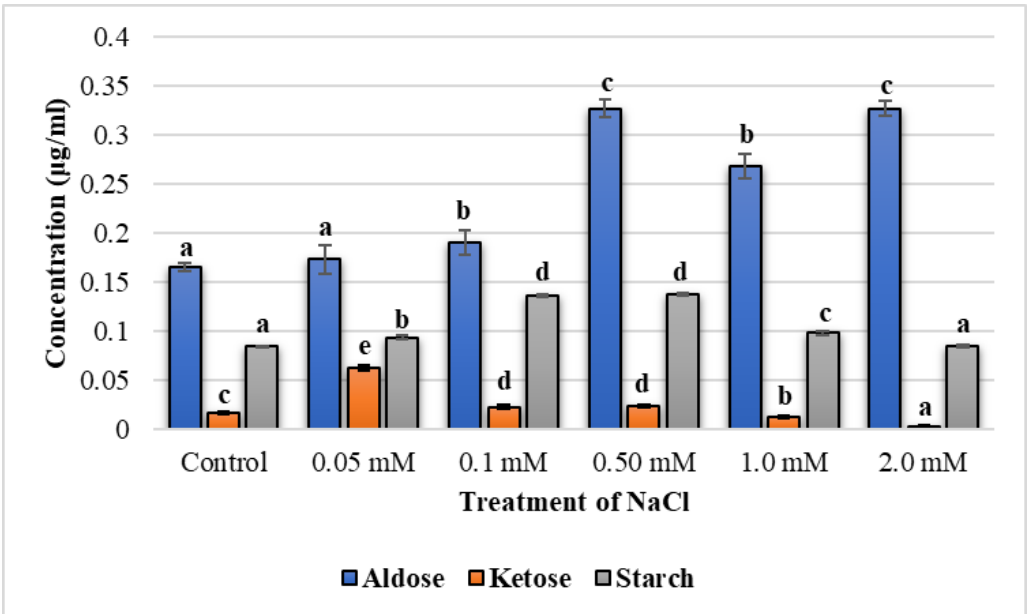

Figure 2. The quantity of total aldose, ketose and starch content present in *A. annua* at 90 DAP are affected by varying NaCl concentrations. Data were represented as a mean ± SD of triplicates of each treatment are labeled with identical superscript alphabets are not significantly different (Duncan's Test, $p \leq 0.05$).

2.7. Antioxidant activities

Plants have developed a highly regulated mechanism that governs the function and production of antioxidant enzymes (namely catalase and TBARS). Catalase activity was enhanced with an increase in NaCl; it was on the higher side at 0.1 mM. When plants were treated with 0.05 mM and 0.1 mM NaCl, an increasing trend was observed, which slowly declined as the dose was increased. However, the catalase activity was comparatively higher compared to the control. At a concentration of 0.05 mM and 0.1 mM of NaCl, the activity of catalase increased by about 200%, which later declined to 52% at a concentration of 2 mM as compared to the control. The measurement of TBARS content was conducted in order to assess the level of oxidative stress and membrane damage in *A. annua*. It was observed that the TBARS content exhibited a progressive increase in response to the varying concentrations of NaCl. Similarly, the TBARS content also showed a hike in activity at the initial increasing doses of NaCl (0.05 mM to 0.50 mM) by more than 100% as compared to the control. The highest activity of 158% was obtained at a dose of 0.1 mM (Figure 3).

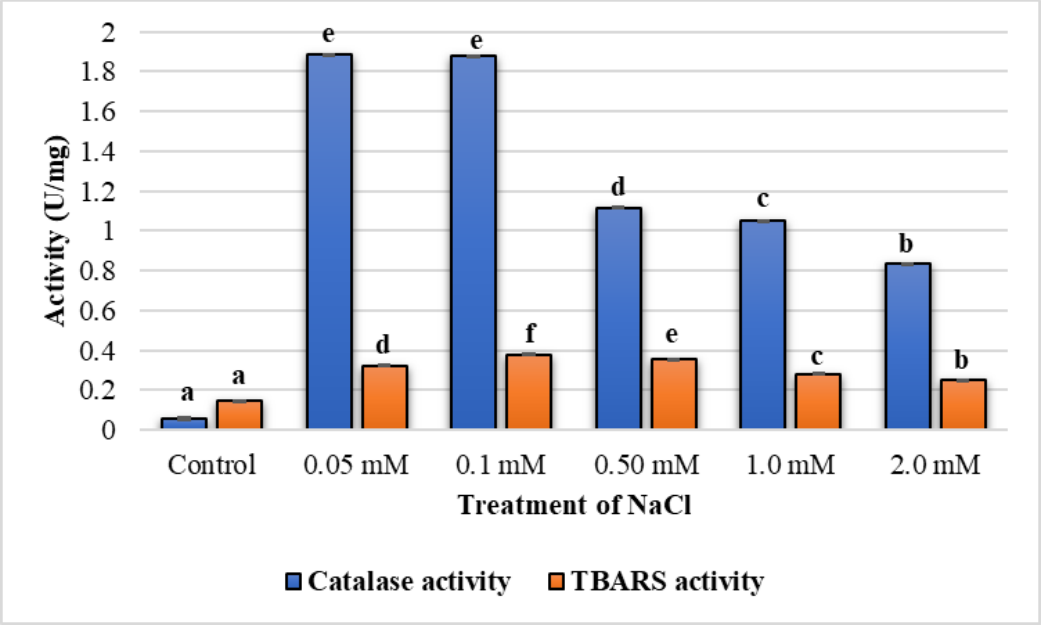

Figure 3. The effect on the catalase and TBARS activity at a different concentration of NaCl present in *A. annua* at 90 DAP. Data were represented as a mean ± SD of triplicates of each treatment are labeled with identical superscript alphabets are not significantly different (Duncan's Test, $p \leq 0.05$).

2.8. Artemisinin concentration determination using HPLC

Increased artemisinin content was observed in *A. annua* at doses of 0.05 mM, 0.1 mM, and 0.5 mM (31.91%, 32.15%, and 116.61%, respectively) as compared to the control. However, as the dose of NaCl increased further, a sharp decline in the artemisinin content was recorded. At concentrations of 1 mM and 2 mM, the concentration reduced by 40.89% and 58.67%, respectively (Figure 4). The data indicates that at an optimal concentration of NaCl, the production of artemisinin can be significantly enhanced. However, if that concentration is increased above the threshold value, it increases the toxicity of the plant, impacting artemisinin production.

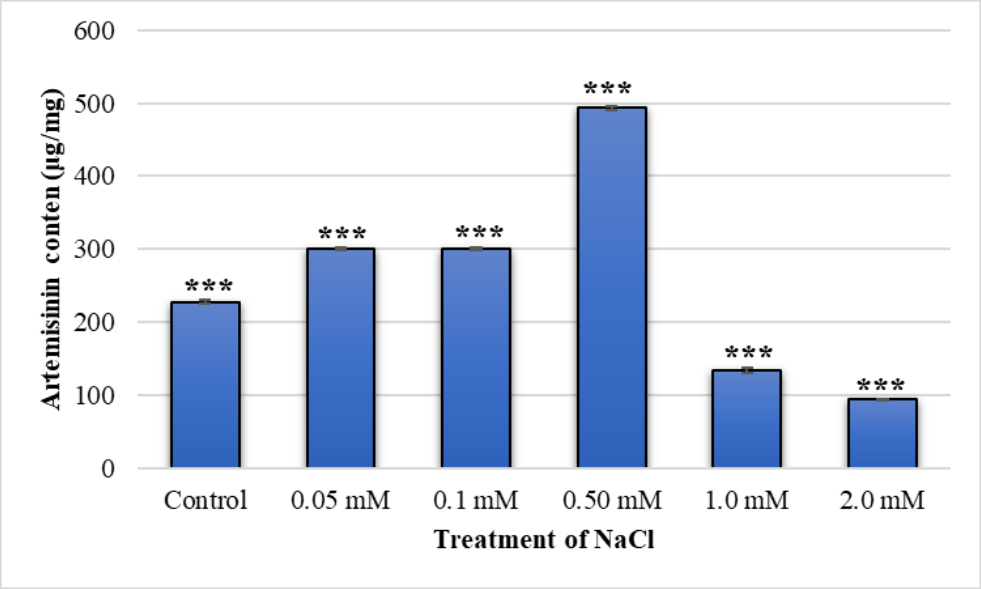

Figure 4. The artemisinin content present in *A. annua* at 90 DAP are affected by varying NaCl concentrations. Data were represented as a mean ± SD (***p ≤0.001).

3. Discussion

Various aspects of *A. annua's* plant development was negatively impacted by exposure to NaCl stress. Total plant biomass, height, leaf biomass, plant canopy, internodal spacing, and branch count are some of these elements. Similar to other abiotic stressors, the effect of salt stress on *A. annua* development causes a decline in the rate of growth. According to Zhao *et al*. (2020), an adaptive mechanism is triggered that helps the plant survive under stressful circumstances. According to Hannachi *et al*. (2022), many plants may restrict their development as a tactical move to enhance their probability of survival. A decrease in photosynthetic activity was reported to be associated with the stunted growth in response to the NaCl-induced stress. The plants were exposed to various concentrations of NaCl salt treatment, and the amount of chlorophyll was significantly decreased (38.01% from 7.91%) as the increased salt concentrations inside the plant may interfere with the production of chlorophyll (Waqas *et al*., 2019; Alharbi *et al*., 2022).

The phenomenon of salt tolerance is frequently observed through discernible alterations in the rate of growth or productivity, which become particularly apparent when exposed to salinity levels that are moderate to low in intensity (Hasanuzzaman and Fujita, 2022). The present study's results are in accordance with the aforementioned concept, as a progressive decline in biomass dry weight was noted in response to escalating salinity levels (as depicted in Table 1). The findings presented here align with the conclusions drawn from the research conducted by Paul and Shakya (2013). In a study, it was observed that plants exposed to a concentration of 4 g/l of NaCl demonstrated a notable reduction in biomass dry weight when compared to the control group. Moreover, the findings of our investigation have revealed a noteworthy reduction in SDW when exposed to the most elevated concentrations (1 and 2 mM). Interestingly, the same treatment provides an increase in the LDW, exhibiting a two-fold increase. Furthermore, an enhanced level of chlorophyll content was also produced at different concentration of NaCl as compare to the untreated plant. The observed inhibited growth in *A. annua* plants can be attributed to the presence of increased levels of salt in the soil, which cause toxicities when accumulated but have the least toxicity to leaves upon accumulation. The experimental findings revealed that the most pronounced adverse effects were observed when the dosage reached its highest concentration of 2 mM. In our investigations, we also observe the elevation of various biochemical elements like total soluble proteins, aldose, ketose and starch at the initial concentration of the NaCl. However, as compare to the control the biochemical compounds level were significant high at a specific concentration of NaCl at 90 DAP.

The presence of increased concentrations of phenolic compounds is reported to be responsible for mitigating the augmented generation of reactive oxygen species (ROS) induced by NaCl stress. The higher level of lipid peroxidation, which is shown by the higher percentage of malondialdehyde, is a sign of oxidative stress in plants that have been exposed to NaCl salinity treatment. The association of malondialdehyde with oxidative

stress has been well documented in the literature (Corrêa-Ferreira *et al*., 2019; AbdElgawad *et al*., 2016). Catalase, an enzyme of significant importance, assumes a central role in the realm of primary antioxidant enzymes, as it is primarily tasked with the crucial responsibility of scavenging reactive oxygen species (ROS) (Li *et al*. 2014). The initiation of lipid peroxidation in cellular systems can occur via two distinct mechanisms: enzymatic and non-enzymatic. The lipoxygenase enzyme is responsible for enzymatic initiation, while free radicals (ROS) help with non-enzymatic initiation. Due to the activation of protective metabolic responses, it is interesting to note that under NaCl salinity stress, the levels of total ROS are elevated, which implies that the impact of NaCl continues to exert its influence to a certain degree (Yadav *et al*., 2015; Yadav *et al*., 2017). In the range of 0.05 to 2 mM of NaCl, the catalase and TBRAS activity was observed to be increasing at lower concentration with a significant level as compare to the control (Figure 3).

NaCl stress has been observed to elicit a notable augmentation in the accumulation of terpenoidal secondary metabolites, like artemisinin, which are derived from glandular trichomes (GT). Numerous investigations have been conducted to examine the behavioural patterns of artemisinin under the influence of abiotic stress factors like salinity and drought (Wani *et al*., 2021). Qian *et al*. (2007) documented the influence of varying levels of NaCl treatment (2 to 8 g/l) administered over a duration of two days on seedlings of *A. annua*. The findings revealed a substantial increase in the artemisinin yield within the seedlings as a result of the aforementioned treatment.

Furthermore, Qureshi and colleagues (2005) reported a comparable finding regarding the enhanced level of artemisinin in *A. annua* subsequent to subjecting the plants to a 10-day treatment of NaCl. The study unveiled that this augmentation manifested in a dose-dependent manner. It is worth mentioning that previous studies examining the influence of NaCl salt on the accumulation of artemisinin in Artemisia annua have primarily focused on short-term stress evaluations, typically ranging from 2 to 10 days. The study was also conducted to determine the effects of NaCl-mediated stress on the A. annua during its life span after two months of differential treatment (Qian *et al*., 2007; Qureshi *et al*., 2005). Similarly, our NaCl mediated stress also influence the increased synthesis of artemisinin at an optimal concentration of NaCl as compare to the control. However, the induced toxicity was also detected as it interferes the elevated production of artemisinin at higher dose of NaCl. Despite the fact that Qian *et al*. (2007) said that artemisinin accumulation per unit weight was stimulated under NaCl stress, they also noted a drop in biomass, which affirms the current study's findings. Hence, these studies help in determining the threshold concentration of NaCl to enhance the yield of artemisinin significantly without the use of heavy toxic metals or biotransformation procedures.

4. Conclusion

The *A. annua* plant exhibited a notable response to NaCl-induced stress; however, it was observed that the highest concentration of NaCl had the most detrimental effect on the growth of the plant. Furthermore, the intermediate dose of NaCl has significantly increased the physiological and biochemical attributes of the plant, thereby indicating that NaCl serves as a mediator of oxidative stress. Interestingly, there is a significant increase in both the concentration and production of artemisinin when exposed to the optimal concentrations of NaCl. This investigation has provided encouraging outcomes, indicating that *A. annua* demonstrates promising potential for experimentation in real field conditions when subjected to salt stress. The exploration of underutilised saline soils presents a promising avenue for harnessing their potential in the cultivation and utilisation of the highly valuable and in-demand antimalarial drug compound, artemisinin.

Statements and Declarations

Acknowledgement: The authors acknowledge the Office of Doctoral Studies and Research for critically reviewing the manuscript and providing the manuscript number (IU/R&D/2023-MCN0001850).

Funding: None

Competing interests: The authors declare that they have no conflicts of interest.

Ethical approval: Not required.

Availability of Data and Materials: Data can be made available upon request.
Code Availability: None

References

1. AbdElgawad, H., Zinta, G., Hegab, M. M., Pandey, R., Asard, H., & Abuelsoud, W. (2016). High Salinity Induces Different Oxidative Stress and Antioxidant Responses in Maize Seedlings Organs. *Frontiers in plant science*, *7*, 276. https://doi.org/10.3389/fpls.2016.00276
2. Acosta-Motos, J. R., Ortuño, M. F., Bernal-Vicente, A., Diaz-Vivancos, P., Sanchez-Blanco, M. J., & Hernandez, J. A. (2017). Plant responses to salt stress: adaptive mechanisms. *Agronomy*, *7*(1), 18. https://doi.org/10.3390/agronomy7010018
3. Aftab, T., Khan, M. M. A., da Silva, J. A. T., Idrees, M., Naeem, M., & Moinuddin. (2011). Role of salicylic acid in promoting salt stress tolerance and enhanced artemisinin production in *Artemisia annua* L. *Journal of plant growth regulation*, *30*, 425-435. https://doi.org/10.1007/s00344-011-9205-0
4. Ahmad, R., Hussain, S., Anjum, M.A., Khalid, M.F., Saqib, M., Zakir, I., Hassan, A., Fahad, S., & Ahmad, S. (2019). Oxidative stress and antioxidant defense mechanisms in plants under salt stress. *Plant abiotic stress tolerance: Agronomic, molecular and biotechnological approaches*, 191-205.
5. Alharbi, K., Al-Osaimi, A. A., & Alghamdi, B. A. (2022). Sodium Chloride (NaCl)-Induced Physiological Alteration and Oxidative Stress Generation in *Pisum sativum* (L.): A Toxicity Assessment. *ACS omega*, *7*(24), 20819–20832. https://doi.org/10.1021/acsomega.2c01427
6. Al-Khayri, J.M., Sudheer, W.N., Lakshmaiah, V.V., Mukherjee, E., Nizam, A., Thiruvengadam, M., Nagella, P., Alessa, F.M., Al-Mssallem, M.Q., Rezk, A.A., & Attimarad, M. (2022). Biotechnological approaches for production of artemisinin, an anti-malarial drug from *Artemisia annua* L. *Molecules*, *27*(9), 3040. https://doi.org/10.3390/molecules27093040
7. Ashenafi, E. L., Nyman, M. C., Shelley, J. T., & Mattson, N. S. (2023). Spectral properties and stability of selected carotenoid and chlorophyll compounds in different solvent systems. *Food Chemistry Advances*, *2*, 100178. https://doi.org/10.1016/j.focha.2022.100178
8. Badshah, S. L., Ullah, A., Ahmad, N., Almarhoon, Z. M., & Mabkhot, Y. (2018). Increasing the strength and production of artemisinin and its derivatives. *Molecules*, *23*(1), 100. https://doi.org/10.3390/molecules23010100
9. Cakmak, I., & Horst, W. J. (1991). Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (Glycine max). *Physiologia plantarum*, *83*(3), 463-468.
10. Chandlee, J. M., & Scandalios, J. G. (1984). Analysis of variants affecting the catalase developmental program in maize scutellum. *Theoretical and applied genetics*, *69*, 71-77.
11. Corrêa-Ferreira, M. L., Viudes, E. B., de Magalhães, P. M., de Santana Filho, A. P., Sassaki, G. L., Pacheco, A. C., & de Oliveira Petkowicz, C. L. (2019). Changes in the composition and structure of cell wall polysaccharides from *Artemisia annua* in response to salt stress. *Carbohydrate research*, *483*, 107753.
12. Hannachi, S., Steppe, K., Eloudi, M., Mechi, L., Bahrini, I., & Van Labeke, M. C. (2022). Salt Stress Induced Changes in Photosynthesis and Metabolic Profiles of One Tolerant ('Bonica') and One Sensitive ('Black Beauty') Eggplant Cultivars (*Solanum melongena* L.). *Plants (Basel, Switzerland)*, *11*(5), 590. https://doi.org/10.3390/plants11050590
13. Hasanuzzaman, M., & Fujita, M. (2022). Plant Responses and Tolerance to Salt Stress: Physiological and Molecular Interventions. *International journal of molecular sciences*, *23*(9), 4810. https://doi.org/10.3390/ijms23094810
14. Hodge, J. E., & Hofreiter, B. T. (1962). Phenol-sulfuric acid colorimetric method. *Methods in carbohydrate chemistry*, *1*, 388-389.
15. Li, L., Zhang, H., Zhang, L., Zhou, Y., Yang, R., Ding, C., & Wang, X. (2014). The physiological response of *Artemisia annua* L. to salt stress and salicylic acid treatment. *Physiology and molecular biology of plants : an international journal of functional plant biology*, *20*(2), 161–169. https://doi.org/10.1007/s12298-014-0228-4
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. The Journal of biological chemistry, 193(1), 265–275.
17. Ma, N., Zhang, Z., Liao, F., Jiang, T., & Tu, Y. (2020). The birth of artemisinin. *Pharmacology & therapeutics*, *216*, 107658. https://doi.org/10.1016/j.pharmthera.2020.107658
18. Munir, N., Hasnain, M., Roessner, U., & Abideen, Z. (2022). Strategies in improving plant salinity resistance and use of salinity resistant plants for economic sustainability. *Critical Reviews in Environmental Science and Technology*, *52*(12), 2150-2196. https://doi.org/10.1080/10643389.2021.1877033
19. Naeem, M., Aftab, T., Ansari, A. A., Shabbir, A., Khan, M. M. A., & Uddin, M. (2019). Arsenic exposure modulates physiological attributes and artemisinin biosynthesis in *Artemisia annua* L. *Int J Herb Med*, *7*(2), 19-26.

20. Paul, S., & Shakya, K. (2013). Arsenic, chromium and NaCl induced artemisinin biosynthesis in *Artemisia annua* L.: a valuable antimalarial plant. *Ecotoxicology and environmental safety*, *98*, 59–65. https://doi.org/10.1016/j.ecoenv.2013.09.025
21. Prud'homme, M. P., Gonzalez, B., Billard, J. P., & Boucaud, J. (1992). Carbohydrate content, fructan and sucrose enzyme activities in roots, stubble and leaves of ryegrass (Lolium perenne L.) as affected by source/sink modification after cutting. *Journal of Plant Physiology*, *140*(3), 282-291. https://doi.org/10.1016/S0176-1617(11)81080-1
22. Qian, Z., Gong, K., Zhang, L., Lv, J., Jing, F., Wang, Y., Guan, S., Wang, G., & Tang, K. (2013). A simple and efficient procedure to enhance artemisinin content in *Artemisia annua* L. by seeding to salinity stress. *African Journal of Agriculture*, *1*(4), i+-46.
23. Qureshi, M. I., Israr, M., Abdin, M. Z., & Iqbal, M. (2005). Responses of *Artemisia annua* L. to lead and salt-induced oxidative stress. *Environmental and Experimental Botany*, *53*(2), 185-193. https://doi.org/10.1016/j.envexpbot.2004.03.014
24. Roe, J. H. (1934). A colorimetric method for the determination of fructose in blood and urine. *Journal of Biological Chemistry*, *107*(1), 15-22.
25. van der Pluijm, R. W., Amaratunga, C., Dhorda, M., & Dondorp, A. M. (2021). Triple artemisinin-based combination therapies for malaria–a new paradigm?. *Trends in parasitology*, *37*(1), 15-24. https://doi.org/10.1016/j.pt.2020.09.011
26. Wani, K. I., Choudhary, S., Zehra, A., Naeem, M., Weathers, P., & Aftab, T. (2021). Enhancing artemisinin content in and delivery from *Artemisia annua*: a review of alternative, classical, and transgenic approaches. *Planta*, *254*(2), 29. https://doi.org/10.1007/s00425-021-03676-3
27. Waqas, M. A., Kaya, C., Riaz, A., Farooq, M., Nawaz, I., Wilkes, A., & Li, Y. (2019). Potential Mechanisms of Abiotic Stress Tolerance in Crop Plants Induced by Thiourea. *Frontiers in plant science*, *10*, 1336. https://doi.org/10.3389/fpls.2019.01336
28. Yadav, R. K., Sangwan, R. S., Srivastava, A. K., & Sangwan, N. S. (2017). Prolonged exposure to salt stress affects specialized metabolites-artemisinin and essential oil accumulation in *Artemisia annua* L.: metabolic acclimation in preferential favour of enhanced terpenoid accumulation accompanying vegetative to reproductive phase transition. *Protoplasma*, *254*(1), 505–522. https://doi.org/10.1007/s00709-016-0971-1
29. Yadav, R. K., Sangwan, R. S., Srivastava, A. K., Maurya, S., & Sangwan, N. S. (2015). Comparative profiling and dynamics of artemisinin related metabolites using efficient protocol and expression of biosynthetic pathway genes during developmental span of two elite varieties of *Artemisia annua* L. *Journal of Plant Biochemistry and Biotechnology*, *24*, 167-175.
30. Zhao, C., Zhang, H., Song, C., Zhu, J. K., & Shabala, S. (2020). Mechanisms of plant responses and adaptation to soil salinity. *The innovation*, *1*(1). https://doi.org/10.1016/j.xinn.2020.100017