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The effect of the combined application of elicitors to *Salvia virgata* Jacq. under salinity stress on physiological and antioxidant defense

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Abstract

Salinity stress is one of the most important stress barriers to crop production worldwide. Developing and implementing new strategies against salinity stress is critical for increasing agricultural productivity and supporting sustainable farming. Elicitors such as nanoparticles and Salicylic acid have recently been used potentially for better product yield. Therefore, in our research the *Salvia virgata* plant was exposed to salinity (NaCl) stress, and zinc oxide nanoparticles (ZnONP), salicylic acid (SA), and the ZnONP + SA combination were applied to plants divided into different groups. While salinity stress decreased the amount of chlorophyll *a*, chlorophyll *b*, and carotenoid pigments, SA, ZnONP, and SA + ZnONP elicitors combined with salinity stress enhanced the content of all three pigments. While salt stress raised MDA, H_2O_2, total phenolic, total flavonoid, soluble sugar and proline content, elicitor applications enhanced proline, soluble sugar, total phenolic and total flavonoid content more. Additionally, the application of NaCl + SA + ZnONP increased proline content by 21.55% and sugar content by 15.73% compared to NaCl application, while decreasing MDA content by 42.28% and H_2O_2 levels by 42.34%, thereby alleviating the plant's salt stress. It was revealed that DPPH, ABTS, and CUPRAC antioxidant activity sequence used to determine the total antioxidant activity displayed similarities, and it was found as NaCl + ZnONP > NaCl + SA > NaCl + SA + ZnONP > NaCl > Control. Furthermore, all elicitor applications increased CAT, GR, APX, and SOD enzyme activities while reducing oxidative stress in *S. virgata* plants. When all the data were evaluated, it was confirmed that SA and ZnONP had a synergistic effect and that SA and ZnONP have the potential to support plant development and growth under salinity. SA and ZnONP applications may have the capacity to least the detrimental impacts of salinity stress on plants. However, further research is needed to investigate the effectiveness of SA and ZnONPs in ameliorating salinity or different stress factors in various other plants.

Keywords Antioxidant, Salicylic acid, Salinity stress, Zinc oxide nanoparticles, *Salvia virgata*

Introduction

Climate change is increasingly threatening agricultural production by increasing the frequency and severity of both biotic and abiotic stress conditions. Drought and salinity are two major abiotic factors that affect plant growth, development, and ultimately its yield [1]. These abiotic stresses limit the worldwide utilization of arable lands and negatively affect crop productivity. Therefore, understanding how plants perceive stress signals and adapt to adverse environmental conditions is critical for

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global food security [2]. Salinity stress is one of the most important stress factors in the globe, lowering crop yield and production dramatically, especially in arid and semi-arid areas [3] Salinity stress is first sensed by the root system and induces osmotic stress caused by reduced water availability. Ultimately, it produces nutritional imbalance due to ion toxicity in the cytosol and hinders plant growth in the long run [4]. As a result, the two major hazards posed by salinity are osmotic stress and ion imbalance caused by extreme sodium (Na^+) and chlorine (Cl^-) ingestion, which causes other nutrient imbalances, particularly calcium (Ca^{2+}) and potassium (K^+). Plants exposed to salinity stress had lower leaf area, leaf number, plant height, fresh-dry weight and root-shoot ratio [5, 6].

Plants use osmotic signaling pathways to regulate processes ranging from osmolyte accumulation to gene expression to alleviate the osmotic stress caused by salinity stress [7]. Plant cells store carbohydrates like sucrose, sorbitol, and mannitol, nitrogen compounds like betaine, proline, and putrescine, and organic acids like malate and oxalate, which help to regulate the osmotic potential against salt stress [8]. Whereas salinity affects photosynthesis via stomatal restrictions, resulting in a decrease in carbon uptake, it may also affect the photosynthetic process via a drop in chlorophyll and carotenoid content [9]. Secondary stressors, such as oxidative stress caused by salinity, are frequently generated by an excess of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl (OH•) and the superoxide radical (O_2•-). ROS, which is plentiful and detrimental because of oxidative stress, increases membrane fluidity and permeability by triggering lipid peroxidation [10], causing denaturation of functional and structural proteins, disruption of photosynthetic pigments [11], and adverse impacts on nucleic acids through base modifications [12]. Plant cells under stress, enzymatic (e.g. catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD) and ascorbate peroxidase (APX)) and non-enzymatic (e.g. carotenoids, α-tocopherol, reduced glutathione (GSH), ascorbic acid (AA), phenolics, proline) decrease the harmful consequences of ROS with antioxidant defense mechanisms [13].

Elicitors are biotic and abiotic compounds that can trigger plant responses to stress and enhance the biosynthesis of secondary plant metabolites. Among abiotic elicitors are physical factors (UV, ozone, etc.), chemical factors (heavy metals, nano-oxides, etc.), and hormonal factors (jasmonic acid, salicylic acid, etc.). Biotic elicitors are biological substances containing polysaccharides and pathogens (yeast, bacterial, and fungal extracts) [14, 15]. Elicitor applications are among the strategies developed to eliminate or minimize the risks posed by biotic and abiotic stresses on plants. Elicitors represent chemical or bio-factors from various sources that can stimulate phytoalexin accumulation with physiological and morphological responses in target living organisms [16]. Nanoparticles (NPs) included in this scope consist of very small-sized components with a diameter ranging from 1 to 100 nm, and these components have numerous significant impacts [17]. There is literature indicating that nanoparticle and salicylic acid applications are effective strategies in combating salinity stress. We can summarize some of these studies and their findings as follows. Under salinity stress in many plants, certain NPs have been found to increase enzymatic and non-enzymatic antioxidant activity, proline, phenolic content, and decrease MDA and hydrogen peroxide (H_2O_2) content [18, 19]. Salicylic acid (SA) is a inherently occurring simple phenolic chemical that acts as a signaling molecule, influences many biochemical and physiological activities, and increases resistance to environmental stimuli [20]. According to reports, exogenous SA application as an elicitor in various plants exposed to salinity stress improves oxidative stress [21], increases proline content [22], increases the pigment content, and reduces MDA content [23].

Salvia is an important genus with high economic value within Lamiaceae family that comprises more than 1000 species. Salvia species have been known as interesting medicinal plants with medical and flavoring properties, and nowadays the essential oils of the various species of this genus are extensively used in the pharmaceutical, perfumery, cosmetic, and food industries [24, 25]. *Salvia virgata* is a perennial plant inherent in Asia and southeastern Europe. *Salvia virgata* Jacq, also known as "fatmaanaotu" or "yılancık" in Turkey, is a herbaceous perennial plant that grows to a height of 30–100 cm [26]. This species has been stated to have antimicrobial [27] and antioxidant [28] properties.

Based on literature data, it is understood that the use of elicitors such as nanoparticles and salicylic acid improves the physiological and biochemical responses of plants exposed to salinity stress. Consequently, the application of compounds like nanoparticles and salicylic acid against salinity stress in plants may provide potential benefits for enhancing plant health and productivity. However, these applications need to be carefully evaluated based on plant species and growing conditions. Despite multiple studies on the impact of SA and zinc oxide nanoparticles (ZnONP) on physiological responses in various plants exposed to stress, there are few studies in the literature on the co-use of both and their potential synergistic effects. Furthermore, no study investigating the potential synergistic effect of ZnONP and SA against salinity stress in Salvia species was found. The aim of this

work was to determine the potential impact of ZnONP/SA on the biocehemical, physiological and antioxidant capacity of the plant by applying ZnONP/SA separately and in combination to the *Salvia virgata* plant exposed to salt stress (NaCl). Another aim of our research is to provide foundational knowledge and serve as a valuable reference for future studies.

Materials and methods

Experimental conditions

S. virgata was collected and identified by Assoc. Prof. Dr. Hüseyin EROĞLU in İpekyolu district of Van province in 2021, at an altitude of 1845 m. Sterilized mature *S. virgata* seeds were sown in equal numbers in pots containing a soil:peat:perlite (1:1:1) mixture and watered with ¼ Hoagland's nutrient solution for 4 weeks in a plant growth room with controlled conditions, by being irrigated until they reached field capacity. At the end of this period, seedlings irrigated with ¼ Hoagland's nutrient solution containing 100 mM salt (NaCl) for additional 2 weeks were subjected to NaCl stress. After salinity stress application, intervention groups were formed by applying 500 µM SA and 20 mg/L ZnONP separately and together through exogenous foliar spraying every other day for 1 week. The plants irrigated at the same time and extent with ¼ Hoagland's nutrient solution not containing NaCl were determined as the control group. Thus, plants were divided into five groups: "Control (T_1), 100 mM NaCI (T_2), 100 mM NaCI + 500 µM SA (T_3), 100 mM NaCI + 20 mg/L ZnONP (T_4), and 100 mM NaCI + 500 µM SA + 20 mg/L ZnONP (T_5)." One week after SA and ZnONP application, all plants were harvested, and some of them were powdered in liquid nitrogen and kept at -80 °C, while the remaining part was left to dry in the shade and at room temperature.

Photosynthetic pigment contents

To determine photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and total carotenoids), 0.25 g fresh leaf samples were obtained, homogenized with 2 mL of 80% acetone, and centrifuged at 5000 rpm for 5 min. The absorbance of the extracts was measured in a UV–Vis spectrophotometer at 663 nm for chlorophyll-*a*, 645 nm for chlorophyll-*b*, and 480 nm for total carotenoids [29].

Proline content

An acidic ninhydrin-based colorimetric test was used to determine proline content [30]. 100 mg of fresh leaf samples were taken, 2 mL of 40% methanol was added to them, and homogenization was ensured. After filtering the resulting homogenate through Whatman No. 1 filter paper, 1 mL of a combination of glacial acetic acid and 6 M orthophosphoric acid (3:2 v/v) and 25 mg of ninhydrin was added to it and incubated at 95 °C for 1 h. Following incubation, 5 mL of toluene was added to the reaction mixture, and its absorbance at 520 nm was measured.

Soluble sugar content

5 mL of 70% ethyl alcohol was added to 50 mg of the dry leaf sample and kept in a hot water bath at 80 °C for 60 min. After centrifuging the samples for 20 min at 5000 rpm, 1 mL of the supernatant, 1 mL of 5% phenol, and 5 mL of concentrated sulfuric acid were mixed, and the absorbance of the combination was detected spectrophotometrically at 490 nm [31].

Malondialdehyde content

2 mL of 5% trichloroacetic acid was added to 100 mg of the fresh leaf sample and homogenized. After centrifuging the homogenate at 12,000 rpm for 20 min at 25 °C, 0.4 L of the supernatant and 0.5% thiobarbituric acid were added to it and maintained in a hot water bath at 95 °C for 1 h. At the end of the incubation period, the reaction mixture was spectrophotometrically measured at 532 nm [32].

H_2O_2 content

We homogenized fresh leaf samples using with 0.1% (w/v) trichloroacetic acid and centrifuged at 12,000 rpm for 15 min at + 4 °C. A mixture of 0.6 mL of supernatant, 0.6 mL of 10 mM potassium phosphate buffer (pH 7.0), and 1.2 mL of 1 M potassium iodide was incubated at 25 °C for 1 h before being measured at 390 nm with a UV–Vis spectrophotometer [33].

Total antioxidant activity

The plant samples, which were left to dry, were powdered and then macerated with ethanol for 24 h. The solvent part of the extracts filtered through the filter paper was removed in the evaporator, and the remaining extract was used for activity determinations. Stock solutions were prepared from the acquired solid extracts at a concentration of 1000 µg/ml for use in the determination of total phenolic, total flavonoid, and antioxidant activity (DPPH, ABTS, and CUPRAC). In the DPPH, ABTS, and CUPRAC procedures, ascorbic acid, BHT, and BHA were used as positive controls.

Total phenolic content

100 µL of the extracts' stock solutions were obtained, and they were incubated at room temperature for two hours with 4.5 mL of distilled water, 100 µL of the Folin-Ciocalteu reagent (FCR), and 300 µL of 2% Na_2CO_3. The absorbance of the reaction mixture was measured in a UV–Vis spectrophotometer at 760 nm at the conclusion

of the period, and the total phenolic content was represented as gallic acid equivalent (GAE) [34].

Total flavonoid content

To 100 µL of the stock plant extract, 4.7 mL of 80% ethanol, 100 µL of 1 M potassium acetate, and 100 µL of 10% aluminum nitrate were added, and the solution was incubated for 40 min after being thoroughly mixed. The absorbance of the combination was measured with a UV–Vis spectrophotometer at 415 nm at the conclusion of this period. The overall flavonoid concentration was reported as quercetin equivalent (QE) [35].

DPPH method

The free radical scavenging activity of the extracts was assessed according to the method of [36] using a stable radical, DPPH (1,1-diphenyl-2-picrylhydrazil). 1 mL of each plant extract prepared at various concentrations (25–250 µg/mL) was taken, 4 mL of 0.1 mM DPPH solution was added to it and incubated in the dark and under room temperature conditions for 30 min. At the end of this period, the absorbance of the mixtures was measured with a UV–Vis spectrophotometer at 517 nm. IC_{50} values were calculated using the percentage of DPPH radical scavenging activity and plant extract concentrations (µg/mL). IC_{50} indicates the amount of extract required to reduce the DPPH radical concentration by 50%.

ABTS method

1 mL of each plant extract prepared at various concentrations (5–80 µg/mL) was taken, 4 mL ABTS•+was added to it, and the mixture was incubated in the dark and at room temperature for 30 min. Afterward, the reaction mixtures were read in a spectrophotometer at a wavelength of 734 nm, and the samples' absorbance values were measured [37]. IC_{50} values were calculated using the percentage of ABTS•+cation radical scavenging activity and plant extract concentrations (µg/mL). IC_{50} indicates the amount of extract required to reduce ABTS•+cation radical concentration by 50%.

CUPRAC method

1 mL of 10 mM copper chloride, 1 mL of 7.5 mM neocuproine, 1 mL of 1 M ammonium acetate, and 1 mL of plant extracts prepared at various concentrations (10–200 µg/mL) were added to the reaction tube. After the mixture was incubated in the dark and at room temperature for 1 h, absorbance measurements were made with a UV–Vis spectrophotometer at 450 nm [38]. $A_{0.5}$ values were calculated using the obtained absorbance values and plant extract concentrations (µg/mL). $A_{0.5}$ refers to the amount of extract required to reduce 50% of the Cu(II)-Nc complex to colored Cu(I)-Nc.

Antioxidant enzymes assays

500 mg of fresh leaf samples were homogenized in 100 mM phosphate buffer (pH 7.0), containing 1% w/v polyvinylpyrrolidone (PVP), 10 mM Na_2EDTA, 10 mM KCl, 1 mM $MgCl_2$, and 2 mM DTT, and the homogenates were centrifuged at 14,000 rpm for a period of 20 min at+4 °C, and the obtained supernatant was used to determine catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and superoxide dismutase (SOD) activities according to [39–41], and [42], respectively. The total soluble protein content used in the calculation of enzymes was determined according to the method of Bradford [43].

Statistical analysis

Statistical analysis was conducted using IBM SPSS 21.0. Each assay was carried out at three replicates. The means of the different treatments were compared using one-way ANOVA, and statistical differences were determined using the Duncan's test. $p \leq 0.05$ was considered statistically significant. The bars in the graph represent the mean plus or minus the standard deviation. The data in the table are presented as Mean ± SD. The levels of significance were represented by different letters. The interpretation of the analysis results was initially conducted by evaluating the increases or decreases of all treatments compared to the control.

Results

Whereas salinity stress reduced the content of chlorophyll *a*, chlorophyll *b*, and carotenoids compared to the control, SA, ZnONP, and SA + ZnONP elicitors combined with salinity stress increased the amount of all three pigments compared to salinity stress alone (Fig. 1). The amount of chl a, chl b and carotenoid were the lowest 0.935 ± 0.036, 0.387 ± 0.014, and 2.938 ± 0.063, respectively, in the control group, while the highest chlorophyll *a* and *b* contents were 1.228 ± 0.029 and 0.459 ± 0.023, respectively, in the NaCl group, and the highest carotenoid content was 3.414 ± 0.097 in the NaCI + SA + ZnONP application.

Salt application and all elicitors applied together with salt increased the content of osmolytes, such as proline and soluble carbohydrates, in comparison with the control group (Table 1). In comparision to the control, proline content increased by 48.10% in the NaCl application, 75.57% in the NaCl + SA application, 72.17% in the NaCl + ZnONP application, and 80.02% in the NaCl + SA + ZnONP application. The rates of increase in soluble carbohydrate content compared to the control group were determined as 8.6% in the NaCl application, 13.7% in the NaCl + SA application, 17.5%

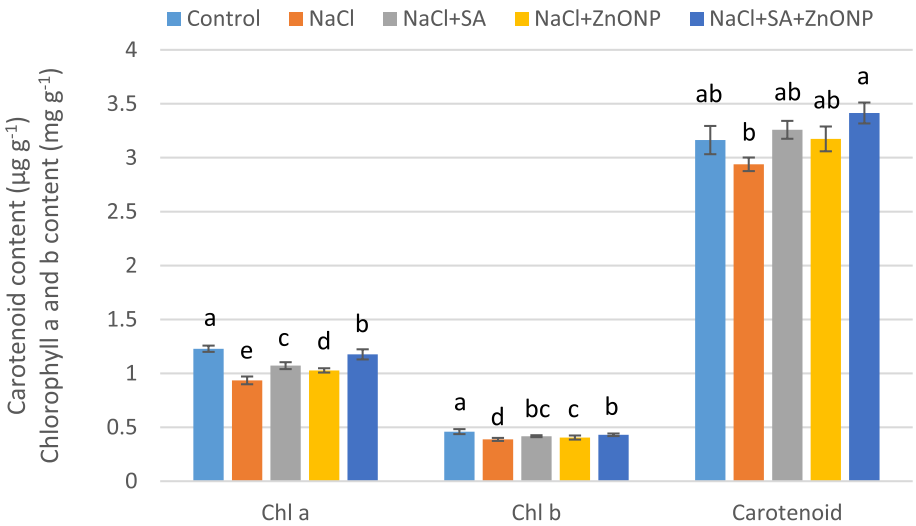

Fig. 1 Effect of salt stress and elicitor applications on photosynthetic pigment contents. The statistical differences were identified using one-way ANOVA and Duncan's test, columns marking the letters were used to signify Mean ± SD and statistically significant variation ($p \leq 0.05$). The study used three biological replicates to ensure accuracy. The carotenoid content is expressed in micrograms per gram (µg g^{-1}), while the contents of chlorophyll a and chlorophyll b are expressed in milligrams per gram (mg $g.^{-1}$)

Table 1 Effect of salt stress and elicitor applications on proline, soluble sugar, MDA, H_2O_2 content

Treatment	Proline (mM g^{-1})	Soluble sugar (mg g^{-1})	MDA (µmol g^{-1})	H_2O_2 (µmol g^{-1})
Control	2.293 ± 0.13^{c}	11.58 ± 0.23^{e}	1.674 ± 0.09^{d}	37.11 ± 1.23^{e}
NaCl	3.396 ± 0.10^{b}	12.58 ± 1.01^{d}	3.666 ± 0.11^{a}	84.22 ± 1.09^{a}
NaCl + SA	4.026 ± 0.11^{a}	13.17 ± 0.85^{c}	2.341 ± 0.05^{c}	55.41 ± 2.37^{c}
NaCl + ZnONP	3.948 ± 0.07^{a}	13.61 ± 0.74^{b}	2.720 ± 0.04^{b}	66.02 ± 1.73^{b}
NaCl + SA + ZnONP	4.128 ± 0.18^{a}	14.56 ± 0.52^{a}	2.116 ± 0.07^{c}	48.56 ± 1.48^{d}

The mean values ± SD from three biological replicates were listed in Table 1, followed by the letters showing statistical differences. One-way ANOVA with Duncan's test ($p \leq 0.05$) was conducted in present research

The superscript letters [a-e] indicate statistically significant differences between applications

in the NaCl + ZnONP application, and 25.7% in the NaCl + SA + ZnONP application. All of the elicitor applications increased proline and soluble carbohydrate contents in comparison with the group to which only salt was applied, and the highest increase was detected for both parameters in the NaCl + SA + ZnONP application.

MDA levels raised in salinity stress and all elicitor applications combined with salt, with the maximum rise rate of 2.18 times recorded in the NaCl application. Furthermore, elicitor applications were found to produce a decrease in MDA content when compared to merely NaCl treatments, with the decrease rate being 25.80% in the NaCl + ZnONP application, 36.14% in the NaCl + SA application, and 42.28% in the NaCl + SA + ZnONP application. Upon evaluating all groups together, it was determined that MDA content was statistically ranked as NaCl > NaCl + ZnONP > NaCl + SA + ZnONP = NaCl + SA > Control. H_2O_2 content increased with both salinity stress and elicitor applications compared to the control, and the highest increase of 2.26 times was observed in the NaCl application. It was found that the elicitor applications caused a reduction in H_2O_2 content compared to only NaCl application and the reduction rate was 21.6% in the NaCl + ZnONP application, 34.2% in the NaCl + SA application, and 42.3% in the NaCl + SA + ZnONP application (Table 1).

An increase was observed in both phenolic and flavonoid contents in salinity stress application and elicitor applications with salt, and statistically significant differences from each other were shown. It was also discovered that elicitor treatments enhanced phenolic and flavonoid content higher than the group that simply received salt stress (Fig. 2). Upon evaluating all groups together in terms of total phenolic content, they were statistically

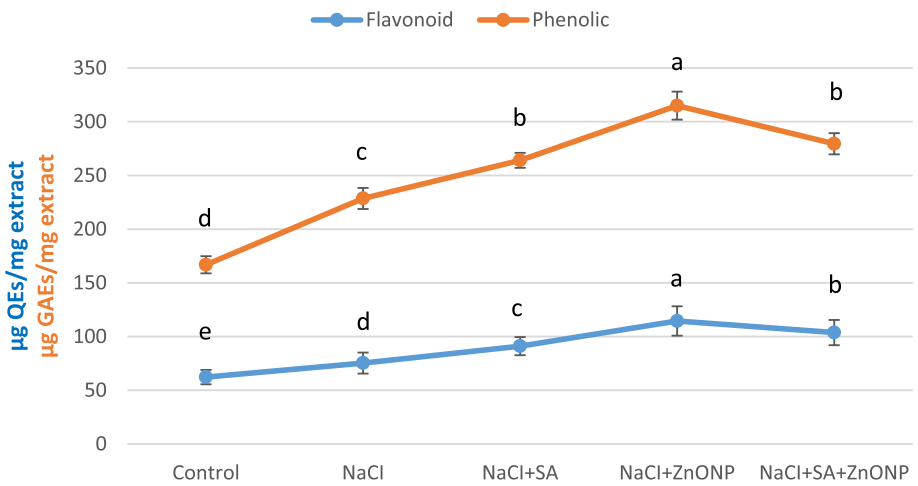

Fig. 2 Total phenolic and flavonoid content of *Salvia virgata* plant extracts. The orange line represents total phenolic content, while the blue line represents total flavonoid content. The statistical differences were identified using one-way ANOVA and Duncan's test, points marking the letters were used to signify Mean ± SD and statistically significant variation ($p \leq 0.05$). The study used three biological replicates to ensure accuracy

ranked as NaCl + ZnONP > NaCl + SA + ZnONP = NaCl + SA > NaCl > Control, and they were ranked as NaCl + ZnONP > NaCl + SA + ZnONP > NaCl + SA > NaCl > Control in terms of the total flavonoid content.

When the IC_{50} values of the total antioxidant activities of the plant extracts were compared, it was discovered that salt application and elicitors combined with salt boosted DPPH, ABTS, and CUPRAC activities in comparison to the control. The highest activity value for DPPH was discovered in the NaCl + ZnONP application (IC_{50} = 87.81 µg/mL), whereas the lowest activity value was reported in the control group (IC_{50} = 117.92 µg/mL). The maximum ABTS activity value was found in the NaCl + ZnONP application (IC_{50} = 21.86 µg/mL), whereas the lowest activity value was found in the control group (IC_{50} = 35.81 µg/mL). When the IC_{50} concentrations of the extract had been compared to those of the positive controls, it was discovered that the positive controls had higher activity than the extracts, although the activity value of the NaCl + ZnONP extract was higher than that of the BHA positive control. The maximum CUPRAC activity value was discovered in the NaCl + ZnONP application ($A_{0.5}$ = 20.46 µg/mL), while the smallest activity value was discovered in the control ($A_{0.5}$ = 74.20 µg/mL). When the $A_{0.5}$ values of the extracts and positive controls were examined, it was discovered that the positive controls had more activity than the extracts, while the NaCl + ZnONP extract had statistically similar activity to BHA (Table 2).

Concerning the antioxidant enzymes' activities, salt application and elicitors applied together with salt induced CAT, GR, APX, and SOD activity were increased in the NaCl + SA + ZnONP application compared to the control, and the maximum induction of all antioxidant enzymes was observed (Fig. 3). The following rates of increase compared to the control were determined: for CAT, 39% in NaCl, 70% in NaCl + ZnONP, 110% in NaCl + SA, and 138% in NaCl + SA + ZnONP; for GR, 49% in NaCl, 123% in NaCl + ZnONP, 161% in NaCl + SA, and 207% in NaCl + SA + ZnONP; for APX, 46% in NaCl, 82% in NaCl + ZnONP, 101% in NaCl + SA, and 140% in NaCl + SA + ZnONP; for SOD, 36% in NaCl, 85% in NaCl + ZnONP, 108% in NaCl + SA, and 217% in NaCl + SA + ZnONP. Upon evaluating all results together, the activity order for the four enzymes was determined as NaCl + SA + ZnONP > NaCl + SA > NaCl + ZnONP > NaCl > Control.

Table 2 Total antioxidant activities of *Salvia virgata* plant extracts

Treatment	DPPH (IC_{50}, µg mL^{-1})	ABTS (IC_{50}, µg mL^{-1})	CUPRAC ($A_{0.5}$, µg mL^{-1})
Control	117.92 ± 0.57^{a}	35.81 ± 0.25^{a}	74.20 ± 0.35^{a}
NaCl	103.64 ± 0.50^{b}	32.58 ± 0.47^{b}	38.33 ± 0.53^{b}
NaCl + SA	92.62 ± 0.55^{d}	26.58 ± 0.55^{d}	30.70 ± 0.24^{d}
NaCl + ZnONP	87.81 ± 0.32^{e}	21.86 ± 0.47^{f}	20.46 ± 0.37^{e}
NaCl + SA + ZnONP	100.67 ± 0.61^{c}	28.76 ± 0.66^{c}	34.70 ± 0.28^{c}
Positive control			
AA	2.76 ± 0.03^{g}	2.17 ± 0.04^{g}	4.06 ± 0.05^{f}
BHT	103.11 ± 0.13^{b}	2.44 ± 0.02^{g}	1.36 ± 0.04^{g}
BHA	17.65 ± 0.07^{f}	22.78 ± 0.12^{e}	20.89 ± 0.09^{e}

The mean values ± SD from three biological replicates were listed in Table 2, followed by the letters showing statistical differences. One-way ANOVA with Duncan's test ($p \leq 0.05$) was conducted in present research

The superscript letters [a-g] indicate statistically significant differences between applications

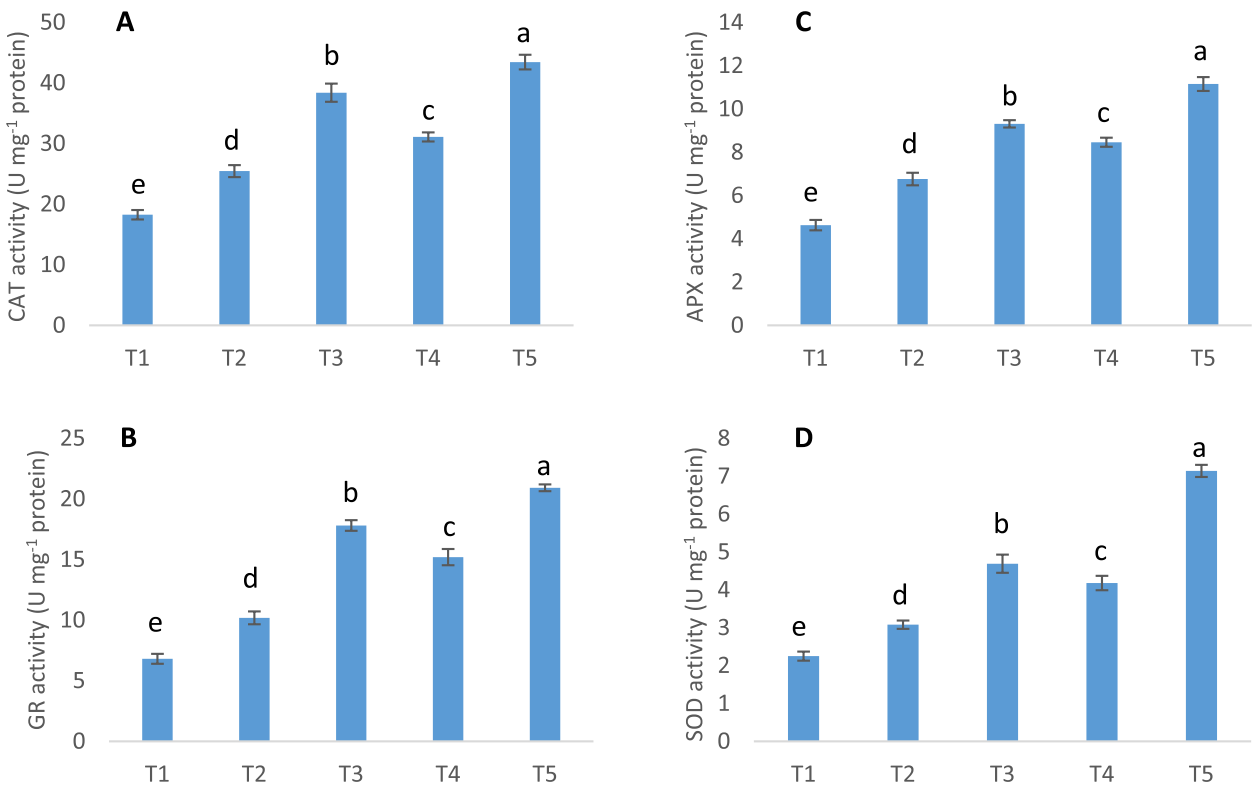

Fig. 3 The effects of salt stress and elicitor applications on CAT (**A**), GR (**B**), APX (**C**) and SOD (**D**) enzyme activities. The statistical differences were identified using one-way ANOVA and Duncan's test, columns marking the letters were used to signify Mean ± SD and statistically significant variation ($p \leq 0.05$). The study used three biological replicates to ensure accuracy

Discussion

Plants have various potential mechanisms to help them cope with the stress effects they are exposed. However, sometimes these mechanisms may be inadequate, leading to the plant's inability to cope with the stress and resulting in plant death. Therefore, researchers are constantly developing different strategies to help plants cope with stress conditions and enhance their tolerance levels against stress. Foliar applications of salicylic acid (SA) and nanoparticles (NP) are among the most suitable methods for stimulating stress tolerance mechanisms against abiotic stress factors in various plants. In the current study, responses of *S. virgata* plants exposed to salinity stress (100 mM NaCl) were investigated through applications of salicylic acid (SA) and zinc oxide nanoparticles (ZnONP), focusing on the plant's reactions to stress and elicitor treatments.

While salinity stress decreased the level of chlorophyll *a*, chlorophyll *b*, and carotenoid pigments in the *S. virgata* plant, SA, ZnONP, and SA + ZnONP elicitors enhanced the concentration of all three pigments. The results indicating that the chlorophyll *a-b* content, which decreased under salinity stress in the Ajwain plant, increased due to SA + nano-Fe_2O_3 applications [44] and the pigment content which decreased with salt in *Salvia officinalis* increased with exogenous SA application [45] are consistent with our research results. Salinity stress damages photosynthetic pigments in plants, resulting in chlorosis, necrosis, and a variety of other significant changes that limit plant growth [46]. It has been stated that the content of aminolevulinic acid (ALA), the precursor of chlorophyll, decreases in leaves under salinity stress [47], enzymes involved in chlorophyll degeneration such as chlorophyllase and pheophorbide monooxygenase increase [48], oxidative stress-induced chlorophyll degradation accelerates [49]. Based on our observations, the decrease in chlorophyll concentration exposed to plants salinity stress might be attributable to an increase in chlorophyll breakdown or a decrease in chlorophyll synthesis. Due to their lipophilic nature and specific properties of capturing peroxyl radicals, carotenoids help prevent cellular damage by reducing the impacts of oxidative stress [50]. In our study, it can be thought that the carotenoid content increasing with salinity stress and elicitors reduces membrane damage by scavenging peroxyl radicals and also reflects on total antioxidant activity.

Plants recover from stress by osmoregulation, which is governed by the accumulation of various osmolytes

such as proline, soluble carbohydrates, and glycine [51]. The increased proline content owing to salt application increased more with SA, ZnONP, and SA + ZnONP treatments, according to our findings about changes in proline and soluble sugar concentrations under salinity stress. The studies reporting that ZnONP applied together with salinity stress increases proline content in *Salvia leriifolia* [52] and MgO-NP in *Daucus carota* [53] the increased proline content in *Mentha pulegium* grown under salinity stress increases more with exogenous SA application [54], and SA and cerium oxide nanoparticle application increases the soluble sugar content in *Mentha spicata* under salinity stress [55] are parallel with our results. Osmoprotectants, such as proline, are critical molecules for salt stress tolerance because they can scavenging ROS and stabilise proteins as well as membranes [56]. Thus, proline is used as a biochemical marker in relation to stress. Soluble carbohydrates play an important role in ROS scavenging, osmotic adjustment, membrane stabilization, and several other vital functions in plants under abiotic stresses [57]. Thus, accumulation of these osmoprotectants is an important defence mechanism in plants which can contribute in regulating turgor and normal cellular metabolism. Whereas soluble carbohydrates function similarly to proline as an osmoprotectant [58] the rise in proline content was higher than soluble sugars in our study. When the plant is stressed by the environment, the benefits of high soluble sugar content include osmotic management, carbon storage, free radical scavenging, membrane protection, and reduced buildup of embedded proteins [59].

Oxidative stress is an important indicator of salt stress, since the plant under the effect of salt may increase the production of ROS and suffer cell damage. H_2O_2 acts as an important signaling factor in cellular defense metabolism at low concentrations, while at high concentrations, it can generate the hydroxyl radical (OH·), which can transpose and degrade cell membranes [60]. Excessive synthesis and accumulation of hazardous substances such as H_2O_2 result in lipid peroxidation and membrane integrity loss, whereas MDA accumulation increases in injured cells [61]. In our research, MDA and H_2O_2 contents increased in salinity stress and all elicitor applications with salt compared to control plants. The increase in MDA [62] and H_2O_2 [63] under salinity conditions is consistent with our study. However, MDA and H_2O_2 levels were lower in the SA, ZnONP, and SA + ZnONP groups compared to the NaCl group. Exogenous SA application reduced MDA content in the radish plant exposed to salinity stress [23], lipid peroxidation level decreased in *Mentha piperita* grown under salinity stress and administered with Fe_2O_3 nanoparticles [64], the application of SA and cerium oxide nanoparticles separately and in combination led to increased peroxidation of membrane lipids and an increase in H_2O_2 content in the *Mentha spicata* exposed to salt stress, and the treatment of SA and cerium oxide nanoparticles reduced both H_2O_2 and MDA contents [55] are similar to our results. Exogenous SA injection boosted the expression of the OsWRKY45 gene, raised endogenous SA content, and lowered H_2O_2 content in stressed *Oryza sativa*, avoiding membrane damage [65]. H_2O_2 functions as a signal molecule and can stimulate gene expression, which is part of the plant's defense system [66].

The reduction of lipid peroxidation and H_2O_2 levels by SA and NP treatments may be connected to the activation of several antioxidant genes that scavenge ROS, according to our findings. Furthermore, increasing nonenzymatic antioxidant content, such as total carotenoid, total phenolic and total flavonoid content, may reduce ROS levels and lead to decreased lipid peroxidation damage in our study.

Plants contain phytochemicals termed as biologically active substances, which include phenolic and flavonoid compounds. These compounds hold significant positions among bioactive compounds, playing crucial roles in numerous biological processes, antioxidant activities, and responses to environmental factors within plants [67]. Only salinity stress and SA, ZnONP, and SA + ZnONP treatments combined with salt increased total phenolic and total flavonoid concentrations as compared to the control group in our study. Furthermore, elicitor treatments enhanced phenolic and flavonoid content more than the group treated just with salt stress. Salinity stress was found to increase the total phenolic content of *Salvia mirzayanii* [68] and the total phenolic and total flavonoid contents of *Salvia lavandulifolia* [69]. The study in which iron oxide nanoparticles were applied to the *Dracocephalum moldavica* plant grown under salinity stress and reporting that the phenolic and flavonoid contents increasing with salinity stress increased more with NP application [18] and the literature data stating that the total phenolic content together with salinity increased more with SA application in the *Vicia faba* plant exposed to NaCl stress [70] are similar to our research results. Polyphenols with several phenolic hydroxyl groups can directly produce active hydrogen and scavenge ROS [71]. Numerous studies have found a relationship between total phenolic content and antioxidant activity in plant extracts [69–72]. These findings support the current study's finding of a relationship among phenolic amount and antioxidant capacity.

To evaluate total antioxidant activity in our investigation, three alternative approaches were used: CUPRAC, DPPH, and ABTS. It was discovered that the antioxidant activity order of the three methods was similar and

in the form of NaCI + ZnONP > NaCI + SA > NaCI + SA + ZnONP > NaCI > Control. The literature reports that salinity stress raised the total phenolic content, total flavonoid content, and DPPH activity in the *Carthamus tinctorius* plant; exogenous SA combined with salt induced a greater rise in all three parameters [73], the total phenolic, carotenoid contents, and DPPH radical scavenging ability increased significantly in *Camelina sativa* grown under salinity stress and treated with ZnONP [74] are similar to our research results. Under environmental stress, both enzymatic and non-enzymatic antioxidant defense systems maintain the balance between detoxification and ROS generation [75]. In our research results, phenolic, flavonoid, and carotenoid contents among non-enzymatic antioxidant compounds generally increased in elicitor applications, and it is seen that the increase in these compounds was reflected in the total antioxidant activity. Exogenously applied elicitors have been found to decrease the impacts of stress by triggering antioxidant enzymatic responses and altering the chemical composition, gene expression, and bioactivity of plant secondary metabolites [76].

Exposure to salinity stress triggers oxidative stress in plants by inducing the production of ROS. Although ROS are involved in plant metabolism, their excessive production under stress conditions can lead to photooxidative damage in various cellular structures. Under stress conditions, plant cells try to survive by using antioxidant enzymes such as catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), and superoxide dismutase (SOD) to mitigate the harmful effects of accumulated ROS on the plant [77]. Foliar SA and SA + ZnONP treatments, in particular, boosted CAT, GR, APX, and SOD enzyme activity and decreased oxidative stress in *S. virgata*. The researchers [78] discovered that CAT, SOD, and GR enzyme activities increased dramatically in the presence of NaCl and ZnONP treatment improved the performance of antioxidative enzymes in *Salvia officinalis* plants. Similarly, applying AgNPs to wheat under salinity stress increased GR, APX, and SOD activities [79], while applying SA to *Mentha pulegium* under salinity stress increased CAT, APX, and SOD activities, mitigating the damaging effects of salt. Our results agree with these reports indicating that SA and ZnONPs increase CAT, GR, APX, and SOD activity together with salinity stress. SA can aid in detoxifying activities under salt by stimulating the specific glutathione S-transferase gene expression [80]. SOD is the first enzyme that deactivates superoxidase radicals to scavenge ROS [81]. GPX competes with APX for H_2O_2 scavenging and serves as the first line of defense against low-level oxidative stress in plants [82]. Under stress, increased antioxidant enzyme activity may be due to increased ROS generation, which activates the plant cell defense mechanism to prevent oxidative damage [83]. SA and ZnONP are observed to induce antioxidant enzyme activity in the *S.virgata* plant.

Conclusion

Globally, soil salinity is an abiotic stress that can threaten arable lands and crop production and consequently can negatively affect food security. Therefore, the aim of this study is to investigate the use of elicitors such as ZnO-NP and SA to reduce the negative effects of salinity stress on crop productivity. The use of elicitors (salicylic acid, jasmonic acid, ethylene, chitin, nitric oxide, nanoparticles, etc.) in plants exposed to stress is an approach aimed at increasing plant resistance through activating their defense responses against stress factors. Although SA and NP applications have the ability to lessen the impacts of stress in plants exposed to salinity stress, their impacts may vary depending on plant type, stress circumstances, application technique, concentration, and frequency of application. Hence, there is a need for further research on SA and NP-based stress management strategies. In our study, the combined application of SA and ZnONP under salt stress conditions has been more effective compared to their individual applications. Compared to salt stress application alone, the SA + ZnONP treatment resulted in the greatest increase in proline and sugar content, and the most significant reduction in MDA and H_2O_2 levels. Additionally, it significantly enhanced the activity of antioxidant enzymes, thereby improving the plant's potential to cope with stress. The SA + ZnONP treatment significantly improved the plant's tolerance to salt stress compared to salt stress application alone, by increasing catalase activity by 70.68%, APX activity by 64.94%, GR activity by 105.29%, and SOD activity by 131.81%. The acquired results demonstrate that elicitors, such as SA and ZnONP, can display synergistic effects and researching the synergistic effects of different elicitor combinations is a potential area of evaluation. These results form an important basis for future research. Investigating the synergistic effects that may occur when several elicitors are combined may lead to the development of more effective ways for increasing plant stress tolerance and optimizing secondary metabolite synthesis. Additionally, the synergistic effects of different combinations of SA and NPs, as well as other intracellular signaling molecules, should be studied under various stress conditions and optimized to meet the needs of plants. Moreover, this research is expected to encourage further studies on innovative methodological applications for preventing salt stress in plants.

Abbreviations

ROS Reactive oxygen species
SOD Superoxide dismutase

CAT	Catalase
GR	Glutathione reductase
APX	Ascorbate peroxidase
NaCl	Sodium chloride
SA	Salicylic acid
ZnONP	Zinc oxide nanoparticle
MDA	Malondialdehyde

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Authors' contributions
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Türkan OKTAY BOZABA] and [İbrahim SELÇUK KURU]. The first draft of the manuscript was written by [İbrahim SELÇUK KURU] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
For this article no studies with human participants or animals were performed by any of the authors. All the experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication
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