Supportive effect of naringenin on NaCl-induced toxicity in *Carthamus tinctorius* seedlings

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Abstract

Background

The exogenous application of priming molecules to plants helps them to develop tolerance against salinity stress. In the present study, we used exogenous naringenin (0.5 mM) pretreatment before the stress in safflower seedlings under 25 mM NaCl to elucidate the role of naringenin to alleviate oxidative conditions associated with salinity complications.

Results

Our results showed biomass, leaf relative water content, chlorophyll content, K^+ content, and K^+/Na^+ ratio were negatively affected by 25 mM NaCl. However, the H_2O_2 accumulation, malondialdehyde (MDA) content, antioxidant enzymes and Na^+ content of NaCl-stressed safflower seedlings were remarkably increased. The results obtained in the present study showed the beneficial effects of the pre-treatment of naringenin in safflower seedlings under non-salinity stress condition with respect to increasing plant biomass, total phenolic compound, radical scavenging activity (RSA), soluble sugar content, proline, glutathione, enzymatic antioxidants, and K^+ content. Also, the results showed that naringenin pre-treatment can (partly) be overcome NaCl-induced stress on safflower seedlings, probably due to higher accumulation of plant biomass, total phenolic compound, RSA, catalase (CAT) activity, and K^+/Na^+ ratio as well as lowering the H_2O_2 and MDA content in the leaves.

Conclusions

Generally, it could be concluded that, pre-treatment of naringenin before stress could partly diminish NaCl-caused oxidative stress in safflower seedlings, probably due to improvement in enzymatic and non-enzymatic antioxidant and reduced cell membrane damage.

1. Introduction

Salinity stress is the main environmental stress and one of the most important environmental factors limiting agricultural production. Salinity is the most drastic abiotic stress, which poses a hazard on crop productivity worldwide and can affect various quantitative and qualitative aspects of plant growth and development (Oommen and Singh, 2002). The area of saline lands in Iran is 15% of the total arable land, which is about 25 million hectares (Mostafazadeh-Fard et al., 2007). In addition, the reduction in the amount of water available to growing plants is one of the most important agricultural problems in Iran (Baghalian et al., 2011). For this reason, the use of water of poor quality in the production of the product is very common. In areas with low vegetation, it is important to pay attention to the use of salinity tolerant species to protect the soil (Ines, et al., 2008). Cultivation of some plants tolerant to salinity stress is a

promising window that, by using them, it is possible to regenerate pastures, develop fields and increase species diversity in natural and agricultural ecosystems. This situation requires a study on the potential of these plants to grow in saline soils and find ways to increase their efficiency in tolerance of water and soil salinity and toxic elements. Safflower (*Carthamus tinctorius* L.) is a plant species from the family of Apiaceae and is considered as an important industrial and medicinal plant. Safflower is widely grown as a source of vegetable oils and carthamine dyes and for nutritional purposes (Sabzalian et al., 2008). *C. tinctorius* is a moderately abiotic stresses-tolerant crop and due to its flexibility to tolerate salt stress by producing antioxidants, including phenolic compounds, safflower is cultivated particularly in semi-arid and arid areas (Karray-Bouraoui et al., 2010). However, salinity stress has become a significant problem in safflower production and management in many areas of the world. Salinity has osmotic, ionic and nutritional constraint effects on plants. These effects lead to growth retardation, metabolic disturbances, and oxidative stress. Plants may tolerate and adapt to these stressors with different mechanisms including changed leaf architecture, osmotic adjustment, ion exclusion, and compartmentalization and more efficient reactive oxygen species (ROS) scavenging systems (Van Zelm et al., 2020).

Salt stress tolerance in plants can be improved by different approaches including the application of organic matter and biofertilizers (Amjad et al., 2015), seeds pre-treatments (Masondo et al. 2018; Abdelhamid et al. 2019), allelochemicals (Niakan et al. 2008) and foliar application of organic and inorganic substances (Jabeen, 2018). In this regard, the exogenous application of priming molecules to plants helps them to develop tolerance against salinity stress. Thus, priming plants with chemical agents appears to be a promising tool to mitigate NaCl-induced growth prevention. Phenolic compounds (as allelochemical), including flavonoids, as the low molecular weight non-enzymatic antioxidants, are broadly distributed in plants and are implicated in many crucial metabolic and physiological processes (Kumar et al., 2019). These compounds play an important role in keeping cellular redox homeostasis and are good candidates for improving plant resistance to stress (Trchounian et al., 2016). Phenolic compounds impact the plant growth and development in higher plants through the mediating auxin transport, scavenging of free radical, accumulation of plant biomass, biosynthesis of photosynthetic pigments, and improvement of plant metabolism (Tohidi et al., 2017; Tanase et al., 2019). They can change some molecular and biochemical processes that allow fighting against salinity-induced oxidative stress by supporting more significant scavenging of free radical and antioxidant activity and inhibition of the lipid peroxidation and stabilization of membranes (Schroeter et al., 2002; Sharma et al., 2019). Naringenin (4',5,7-trihydroxyflavanone), a natural plant flavonoid belonging to the flavanones subclass, is a bioactive compound present in a variety of citrus fruits and has been found to possess potent anti-diabetic, anti-inflammatory and anti-oxidative properties (Salehi et al., 2019). However, the effect of this compound on improving plant yield under stress has been less considered. Some studies have shown that the application of exogenous antioxidant compounds in activating the plant defense system against many stresses such as heavy metals (Sharma et al., 2021; Gokul et al., 2021), cold (Ozfidan- Konakci et al., 2019) and salinity stress (Idrees et al., 2011; Azooz et al., 2013; Liu et al., 2016; Ozfidan-Konakci et al., 2020). Recently, Ozfidan-Konakci et al. (2020) showed the involvement of naringenin in the improvement of growth and increment of osmotic adjustment on salt (100 mM NaCl)-stressed *Phaseolus vulgaris*.

Also, the potential of naringenin in the reduction of Cd-induced toxicity has been reported in *Vigna radiate* (Sharma et al., 2021). Naringenin has been shown to improve vegetative growth, biochemical accumulation, and protein bioavailability in mungbean (Sharma et al., 2020). To date, no studies have investigated the potential of using exogenous naringenin to improve salinity stress resistance in safflower seedlings. Therefore, we conducted this study to assess the effect of exogenous naringenin pretreatment on safflower seedlings under NaCl stress to elucidate the role of naringenin to alleviate oxidative conditions associated with salinity complications.

2. Materials And Methods

Seeds of *Carthamus tinctorius* L. were purchased from Pakan Bazr Company (Isfahan, Iran) and only intact seeds without visible defects were selected. Two preliminary experiments for the determination of ideal experimental concentrations of NaCl and naringenin were applied to seeds of *C. tinctorius*. In the first stage of the preliminary experiment, the effect of various concentrations of NaCl (0, 25, 50, 100, 150, 200, 250, 300 and 350 mM) was measured on seed germination and seedling growth for one week. This stage was conducted using 20 surface- sterilized seeds in 90 mm petri dishes in 3 replicates. Petri dishes were incubated in a growth chamber (25°C, 50% humidity, light for 16 h, and dark for 8 h). In the second stage of the preliminary experiment, sterilized seeds of safflower were primed with various concentrations of naringenin (0.15, 0.35, 0.5, 0.65 and 1 mM) for 8h. The seeds washed rapidly with water then were dried with filter paper under laboratory conditions. Seed germination and seedling growth tests were determined after one week under the same condition mentioned above. In the main experiment, ten surface-sterilized seeds were placed in the 90 mm petri dish (in four replicates) containing 30 ml distilled water and incubated in a growth chamber (25°C, 50% humidity, light for 16h, and dark for 8h) for one week. Then, seedlings for two weeks were transferred to half-strength Hoagland's medium with pH= 5.8. The plants were grown under a photoperiod of 16/8h light /dark and temperature 27 ºC in a greenhouse. After two weeks of acclimatization, the seedlings were divided into two sub-groups for competition under different treatments.

The half of 21-days-old seedlings transferred to fresh Hoagland solution containing naringenin (0.5 mM) for 72h. Naringenin (Alfa Aesar, Thermo Fisher (Kandel) GmbH, Germany; 97%; CAS Number 67604-48-2) was dissolved in distilled water with 5% Tween 80. After naringenin pretreatment, the naringenin removed by washing and changing fresh Hoagland solution. In the second sub-group, seedlings were transferred to fresh Hoagland solution without naringenin (0 mM). Three days after treatment, half of the naringenin-pretreated seedlings and half of the non-pretreated seedlings were separated and irrigated with half-strength Hoagland’s nutrition containing 25 mM of NaCl for one week. The experiment was performed with a randomized complete design with 3 replicates using the following treatments: 1) Control 2) NaCl 3) Naringenin 4) Naringenin+NaCl. Plants were harvested after one week of treatment and then stored at -80°C until further analyses.

2.1. Determination of dry weight, water content, RSA (radical scavenging activity) and proline content

Dry weight analysis was performed by drying seedling shoots (individually) per treatment at 75°C for 48 h and the weights were subsequently recorded. The leaf relative water content (RWC) was calculated by the following formula (Turner, 1986):

RWC (%) = [(FW-DW) / (TW-DW)] x 100

Assessment of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging capacity was performed by preparing 500 µl of sample ethanolic extract in a tube. Then, 500 µl of DPPH solution was added to the extract. The solution was thoroughly mixed and incubated at room temperature for 30 minutes. The absorbance of control ethanol sample (blank), control water sample and samples obtained from plant extracts was read at 520 nm (Kulisic et al. 2004). The capacity of scavenging free radicals of samples was calculated as follows:

% Radical Scavenging Activity=((OD control water –OD Sample)/OD control water)*100

Proline content was measured according to Troll and Lindsley (1955). The leaves were homogenized in 70% ethanol and homogenate was filtered through filter paper. After adding of acetic acid, ethanol, ninhydrin and water, the mixture was heated at 95°C in water bath. Then, the absorbance of the mixture was measured at 520 nm.

2.2. Determination of pigments photosynthetic

Fresh leaf samples (0.1 g) were extracted with 10 ml acetone (80%, v/v). The filtrates were prepared and the absorption values were read at 663, 645 and 470 nm, to assess the contents of chlorophyll *a*, chlorophyll *b* using the method of Arnon (1949) and carotenoids in the extracts using the method of Lichtenthaler (1987).

2.3. Measurement of oxidative stress markers

The hydrogen peroxide (H_2O_2) concentration was extracted and assessed after reaction with potassium iodide (KI) following the method of Alexieva et al. (2001). The malondialdehyde (MDA), glutathione (GSH) level and glutathione reductase (GR) activity in the leaves of safflower was detected by commercially available kits (KIA ZIST, Hamadan, Iran). The MDA levels, GSH levels and GR activity were measured according to the manufacturer's instructions. The absorbance was measured using a spectrophotometer at 532, 405 and 405 nm, respectively.

2.4. Measurement of total phenolic compounds

The total soluble phenolic compounds were estimated according to the procedure defined by Singleton and Rossi (1965). Leaves of safflower seedlings were homogenized in 80% methanol and centrifuged at 12 000g for 15 min. The reaction was initiated by adding 975 µl of 2% Na_2CO_3, 25 µl of Folin–Ciocalteu

reagent and 470 µl distilled water to crude enzyme extracts. The absorbance was measured using a spectrophotometer at 765 nm. The total soluble phenolic compounds were calculated using a standard curve prepared with known concentrations of gallic acid (Sigma-Aldrich).

2.5. Enzyme extraction and determination of CAT, SOD and APX enzyme activities

For enzyme extractions, 0.1 g of each sample was ground to a fine powder using liquid nitrogen and then homogenized in extraction buffer (PBS) pH= 7.8. The catalase (CAT) activity was determined using the method described by Aebi (1984). The reaction was initiated by adding 50 mM phosphate buffer (pH= 7.8), and 20 mM H_2O_2 to crude enzyme extracts. The absorbance was measured using a kinetic spectrophotometer at 240 nm. For APX activity estimation (Nakano and Asada, 1987), the reaction was initiated by adding the leaf extract to a reaction mix comprising phosphate buffer (50 mM, pH=7.8), 0.2 mM EDTA, 50 mM ascorbic acid, and 0.25 mM hydrogen peroxide. The absorbance was recorded at 290 nm. The activity of CAT and APX was stated as units per 1 g of fresh leaves. For SOD activity determination (Beyer and Fridovich, 1987), 50 mM phosphate buffer (pH= 7.8), 13 mM methionine, 0.1 μM EDTA, 75 μM nitro blue tetrazolium, and 2 μM riboflavin was added to enzyme extracts. The reaction mix was illuminated with 20-watt fluorescent tubes and the absorbance was recorded at 560 nm. One unit of enzyme activity is considered for the quantity of the enzyme that impedes the reduction of NBT by 50% in one minute.

2.6. Measurement of soluble sugar content

Following the method of Porter and Villar (1997), briefly, dry leaf (0.1 g) samples were extracted in 5 ml 80% ethanol (v/v). After boiling the extracts and centrifuging them using anthrone reagent, absorption of samples was read at 625 nm.

2.7. Determination of sodium and potassium concentration

The ash sample of shoots and roots were acid digested and filtered to determine Na^+ and K^+ concentration by flame photometer (Corning, UK). Na^+ and K^+ content were recorded in mg/gDW.

2.8. Statistical analysis

The experiments were designed with three replications The one-way ANOVA analyses were done by SPSS 19.0 software and l.s.d. post-hoc testing was applied to compare the means at $P \leq 0.05$.

3. Results

3.1. Effects of naringenin pre-treatment on seedling growth and chlorophyll content

Results related to the first experiment showed that germination percentage, total fresh weight and shoot length of safflower seedlings were significantly affected by NaCl at all applied concentrations in

comparison to the control (Figure. 1). Application of 25 mM NaCl decreased salt tolerance index by 54% in comparison to control, and was selected for the next experiment.

Also, we select the best concentration of naringenin that caused the maximum significant increment in growth parameters (total fresh weight, shoot and root length) of safflower seedlings in comparison to control (Figure. 2). Therefore, the concentration of 0.5 mM of naringenin was added to safflower seedlings alone or in combination with NaCl (25 mM). A reduction in the shoots and roots dry weight by 28% and 32%, respectively, was observed in NaCl-treated seedlings. Conversely, naringenin pre-treatment diminished salinity-induced inhibition of growth components and promoted better growth (Figure. 3a and b). For RWC, in respect to control plants, NaCl treatment considerably decreased the RWC by 28%. Results showed that pretreatment of safflower seedlings with naringenin did not change RWC levels in leaves. Also, we observed no significant difference in RWC in naringenin+NaCl combination treatment when compared to the NaCl-treated seedlings (Figure. 3c). NaCl treatment led to a reduction in total chlorophyll content of 12%; naringenin pretreatment curtailed a significant amount of chlorophyll loss from their tissues, and these plants contained 17% more total chlorophyll content in comparison to the NaCl treatment (Figure. 3d).

3.2. Effects of naringenin pre-treatment on H_2O_2 and Malondialdehyde (MDA) content

To evaluate the ROS accumulation trend under salinity stress and naringenin pretreatment, we examined the generation rates of H_2O_2 in safflower leaves (Figure. 4a). Relative to the control, sharp increases in H_2O_2 production was observed in the leaves upon subjected to salinity stress. Under NaCl stress, H_2O_2 content increased by 2.13 fold compared with the control seedlings. By contrast, pretreatment with naringenin increased the NaCl-stress tolerance of seedlings by reducing the formation rates of H_2O_2 in the leaves by 37%, compared with the NaCl-treated seedlings. In addition, NaCl treatment increased accumulation of MDA content by 47% in the leaves of safflower in comparison to the control seedlings. Naringenin pretreatment was more effective for overcoming severe effects of NaCl stress, as shown by lower MDA concentration (19%) compared to the NaCl-treated seedlings (Figure. 4b).

3.3. Effects of naringenin pre-treatment on RSA, proline, phenolic, glutathione and soluble sugar content

We quantified antioxidant compounds to clarify how naringenin and antioxidants coordinate in order to delete the harmful impacts of NaCl stress. As shown in Figure 5a, NaCl treatment and naringenin pre-treatment increased RSA content by 21% and 29% in leaves of safflower, respectively, in comparison to the control seedling. Furthermore, we observed increases in RSA content in naringenin+NaCl-treated seedlings (13%) when compared to the NaCl treated seedlings. NaCl-stressed safflower seedlings induced elevated proline content that was 4.6 fold greater compared with the corresponding control. In comparison to plants treated with NaCl alone, the naringenin-pretreatment combined with NaCl-stressed seedlings decreased proline content by 31% (Figure. 5b).

The soluble sugar, glutathione and total phenolic contents significantly increased in NaCl stressed seedlings by 2.3 fold, 97%, and 20%, respectively, in contrast to the corresponding control seedlings (Figure. 5c, d, e). Naringenin pretreatment further increased sugar by 2.1,

glutathione by 1.5, and phenolic contents by 2 fold relative to their corresponding control plants. In comparison to plants treated with NaCl alone, the naringenin-pretreatment combined with NaCl-stressed seedlings increased total phenolic content by 1.46 fold.

3.4. Effects of naringenin pre-treatment on antioxidant enzymes

NaCl treatment significantly increased the CAT, GR and APX activities by 57%, 44% and 4.7 fold, respectively. Naringenin pre-treatment significantly increased the activities of CAT, SOD and APX by 2.4 fold, 29% and 3 fold respectively, in safflower leaves compared to the control seedling. While, naringenin pre-treatment did not affect GR activity. Our finding indicated that in seedlings treated with naringenin+NaCl, the activities of GR and APX significantly decreased but the CAT activity was enhanced in safflower leaves compared to the NaCl treatment (Figure. 6a, b, c and d).

3.5. Effects of naringenin pre-treatment on ions analysis

As shown in Table 1, in comparison to the control plants, exposure to NaCl treatment increased the Na^+ content by 45% and 49% but decreased the K^+ content by 36% and 10% in the leaves and roots, respectively. Thus salinity decreased K^+/Na^+ ratio by 56% and 40% in the leaves and roots of safflower, respectively compared to the control plants. Pretreatment with naringenin enhanced the Na^+ content by 25% and the K^+ content by 32% in the roots of safflower compared to the control plants. However, naringenin pretreatment did not change K^+/Na^+ ratio in the leaves and roots of safflower, compared to the control plants. Similarly, a reduction in Na^+ content by 15% and 10% in the leaves and roots, respectively, was observed in naringenin+NaCl treatment in comparison to the NaCl treatment. An increase in K^+ content of 7% in roots of safflower was observed in naringenin+NaCl combination-treated seedlings when compared to the NaCl-treated seedlings. Furthermore, we observed significant increase in K^+/Na^+ ratio of leaves and roots in naringenin+NaCl combination treatments when compared to plants treated with NaCl alone.

Table 1

Effect of salinity (25 mM NaCl), naringenin pretreatment and naringenin+NaCl on the Na^+, K^+ and Na^+/K^+ ratio of leaves and roots of safflower seedlings. Data represent the mean ± SE (n = 3). Different letters show significant (P < 0.05) differences as determined by l.s.d. test, n = 3.

Treatments	Na^+ mg/g (leaves)	Na^+ mg/g (root)	K^+ mg/g (leaves)	K^+ mg/g (root)	K^+/ Na^+ (leaves)	K^+/ Na^+ (root)
Control	1.57 ± 0.07c	3.48 ± 0.01c	53.71 ± 0.41a	112.34 ± 0.04b	34.21 ± 0.41a	32.27 ± 0.41a
NaCl	2.28 ± 0.06a	5.2 ± 0.02a	34.31 ± 0.25b	100.69 ± 0.02c	15 ± 0.05c	19.33 ± 0.15c
Naringenin	1.6 ± 0.03c	4.36 ± 0.02b	54.46 ± 0.29a	148.49 ± 0.01a	34.01 ± 0.29a	34.5 ± 0.29a
Naringenin + NaCl	1.93 ± 0.03b	4.65 ± 0.01b	35.74 ± 0.23b	107.98 ± 0.02b	18.44 ± 0.13b	23.2 ± 0.07b

4. Discussion

Salinity is multifaceted stress with characteristic changes of Na^+ concentration resulting in increased production of ROS. Enhanced ROS give rise to an imbalance between oxidants and antioxidants. When antioxidants are reduced plants normal functions are disturbed and the routine functions of various cellular plant modules are damaged by exposure to ROS. In this present study, we evaluated whether naringenin (0.5 mM) pre-treatment could alleviate some

complications caused by 25 mM NaCl, using safflower seedling compared with that in the non-pretreated seedlings. Under salinity condition, the non-pretreated seedling exhibited significant biomass loss when compared to the control. Growth of the safflower seedlings displayed a marked response towards naringenin pre-treatment. Generally, there was a significant increase in the shoot and root biomass of safflower seedlings pre-treated with naringenin under unstressed condition, and the beneficial effect of naringenin on growth is compromised by NaCl-mediated stress (Figure. 3). One of the initial responses of plants to salinity is inhibition of shoot and root growth. The effects of salinity on alteration in growth might be attributed to changes in water and ion absorption by the roots, production of hormonal signals that exchange messages to the shoot, and changes in gene expression patterns. Several studies have reported the supportive impact of antioxidant compounds on plant growth under stress. The pre-treatment of antioxidant compounds might supply defense opposed to salinity stress in plants. Mekawy et al. (2018) suggested that apigenin pre-treatment improved the growth of rice seedlings by increasing shoot elongation and dry mass accumulation under salinity-stress condition. On the contrary of our results, the exogenously applied flavonoid naringenin can promote RWC (Yildiztugay et al., 2020) and relative growth rate (Ozfidan-Konakci et al., 2020) of *Phaseolus vulgaris* seedlings under NaCl stress. Also, naringenin diminished the cadmium stress and improved shoot-root length and relative water

accumulations in *Vigna radiate* (Sharma et al., 2021) and the root length and fresh weight accumulation in *Arabidopsis thaliana* (Keiling and Ludwig-Mul, 2009). Improving plant biomass by application of antioxidant compounds under salinity stress may be because they act as non-enzymatic antioxidants and are able to scavenge ROS and free radicals generated under stress.

Salinity stress decreased total chlorophyll content in safflower plants, however pre-treatment with naringenin enhanced chlorophyll content under both unstressed and NaCl-stress conditions, and alleviated detrimental effects of salinity. Application of antioxidant compounds play an important role in enhancing chlorophyll content (Singh et al., 2017; Aziz et al., 2017; Sharma et al., 2021). Yildiztugay et al. (2020) showed that naringenin had positive effects on promoting Fv/Fm and Fv/Fo in bean under salinity stress. They attribute this naringenin–induced enhancement to the maintenance of the degraded photosynthetic pigments in the chloroplasts or regulation of the reduction/re-oxidation levels of quinones. Antioxidant compounds can also help plants to retain photosynthetic activity under stress conditions.

The higher production of ROS such as (O_2^-), (OH^-), and H_2O_2 under salinity stress is inevitable. The results showed, as expected, that the H_2O_2 concentration in safflower leaves was enhanced under 25 mM NaCl. Naringenin was able to control the toxic levels of hydrogen peroxide produced by salinity stress in *P. vulgaris* leaves (Yildiztugay et al., 2020). Similarly, pretreatment with naringenin inhibited the H_2O_2 production under salinity stress in safflower seedlings. Abiotic stresses-induced enhancement of ROS results in oxidative stress that causes lipid peroxidation resulting to an overproduction of malondialdehyde (MDA), which is used as a biomarker of lipid peroxidation (Shafiq et al., 2015). MDA levels were significantly enhanced under salinity stress in the un-pretreated safflower seedlings compared to the control (Fig. 3); these results were expected due to NaCl-induced oxidative stress in the safflower seedlings. Naringenin pre-treatment significantly reduced MDA levels under both unstressed and NaCl-stress conditions; these results suggest that naringenin treatment decreased lipid peroxidation by scavenging free radicals reducing oxidative stress (Song et al., 2018). In accordance with our findings, Li et al. (2013) and Yildiztugay et al. (2020) reported that ferulic acid and naringenin pretreatments alleviated the increase in MDA content in PEG-induced dehydration stressed cucumber and salinity-stressed bean, respectively. In this study we found that naringenin did not increase the levels of H_2O_2, however, it elevated the content of MDA as compared to the control. This data indicated that naringenin may induce other free radicles thereby causing oxidative stress or lipid peroxidation. It's possible that naringenin induces free radicles in safflower seedlings other than H_2O_2.

The accumulation of compatible solutes, including proline and soluble sugar was reported under salinity stress likely for modulation of osmotic signalling pathways, stabilization of membranes and action as ROS scavenger (Sharma et al. 2019), as well as facilitation the water absorption (Puniran-Hartley et al., 2014). Salinity stress resulted in a remarkable increase in the proline, soluble sugar content and lowered the water content in leaves, whereas naringenin pretreatment decreased the proline, soluble sugar content and the water content under both unstressed and NaCl-stress conditions in safflower seedlings.

This finding indicates that naringenin unable to help the leaves to maintain a higher water level and lower cellular osmotic potential (Figure. 5), as a result, it does not have played a role on the plant's ability to cope with salinity stress through this defense pathway. Previous studies also showed that application of antioxidant compounds improves salinity and drought stress amelioration in plants by enhancing proline and soluble sugar in rice (Mekawy et al., 2018), and quinoa (Aziz et al., 2018), respectively. It seems that by scavenging ROS, naringenin restrict the continuation of oxidative stress and inhibit the formation of ROS and reducing the cell's need to synthesize proline and soluble sugars, and as a result, cell energy is expended in plant defense pathways against salinity stress instead of costly in the initial metabolism.

Plants to fight against salinity-induced oxidative stress also increased the biosynthesis and accumulation of non-antioxidant compounds, particularly metabolic pathways associated with polyphenolic antioxidants biosynthesis (Cheynier et al. 2013). Results relating to total phenolic compounds and radical scavenging activity (RSA) showed that, salinity stress increased them in safflower plants, indicating that concentrations of these compounds can be regulated by NaCl. Naringenin pre-treatment significantly enhanced the total phenolic compounds and radical scavenging activity under both unstressed and NaCl-stress conditions. Enhancement of in polyphenols contents and RSA of plant tissues by application of antioxidant compounds have been shown in some stressed plants (Bhardwaj et al., 2015, Aziz et al., 2018, Yildiztugay et al., 2018). Mekawy et al. (2018) reported that the apigenin application accumulated significantly higher levels of total flavonoids under unstressed and NaCl stress conditions in rice seedlings. It has been established that naringenin serves as a general precursor of flavonoids in plants; therefore, an increase in polyphenolic compounds in safflower seedlings was expected.

Glutathione (GSH) as a principal cellular redox buffer acts in regulation of cellular thiol redox homeostasis and as a part of signal transduction during environmental, biotic and abiotic stresses (Meyer, 2008), and an important function of this antioxidant compound in protection of cells against stress-induced oxidative stress is the efficient scavenging of overabundance H_2O_2 (Yadav, 2010). Thus, by considering the results of glutathione (Fig. 5d), we found that under salinity stress by pretreatment of safflower seedlings with naringenin, the NaCl-stimulated glutathione contents were significantly reduced, indicating the involvement of naringenin in the ROS detoxification thereby, leading to the reduction harmful effects of salinity on plants and increase their resistance to 25 mM NaCl. According to the results of this study, Yildiztugay et al., 2018 and Yildiztugay et al. (2020), demonstrated that a notable increase in glutathione content occurred with the ferulic acid and naringenin application under stress, respectively.

Antioxidant enzymes such as SOD, CAT and APX and GR are recognized as first-line defense antioxidants, they inhibit the formation of ROS and free radicals, low levels of these antioxidants demonstrate the presence of oxidative stress (Gupta et al., 2018). SOD is a metalloenzyme that catalyzes the dismutation of deleterious superoxide radicals (O_2^-) into O_2 and H_2O_2. The salinity-stressed seedling showed low levels of SOD when compared to control plants (Figure. 6d), which showed that there were increased levels of oxidative stress. Also, these results are consistent with overproduction of H_2O_2 in salinity-stressed seedlings. Naringenin pre-treatment significantly enhanced the SOD levels under unstressed condition,

the reason for the accumulation of the antioxidant enzyme SOD might be the naringenin-induced oxidative stress amelioration.

H_2O_2 generated by SOD, it may be scavenged by CAT, APX (using ascorbate as an electron donor in photosynthetic eukaryotes) and GR into water (Gupta et al., 2018). CAT is a prevalent antioxidant enzyme that uses Fe or Mn as a cofactor and catalyzes the detoxication of deleterious radicals into less deleterious molecules. Under salinity stress, the un pre-treated safflower seedling showed low levels of CAT activity when compared to the control plants (Figure. 6a), this suggests that there were high levels of oxidative stress which is expected for salinity condition. Naringenin pre-treatment significantly enhanced CAT activity compared to the un pre-treated plants under both unstressed and NaCl-stress conditions, these results indicate that naringenin either scavenged free radicals causing reduced consumption of the antioxidants or naringenin could be binding free radicals resulting in less production of the antioxidant enzymes. GR is a vital enzyme for the regulation of cellular redox homeostasis, which also plays a critical role in regeneration of GSH (Couto et al., 2016). Under salinity stress, the un pre-treated safflower seedling showed significantly high levels of APX and GR compared to control (Figure. 6), thus the enhancement of activities of APX and GR had a role in efficient detoxification of extremely produced H_2O_2 in salinity-stressed seedlings. While, naringenin alone did not affect GR activity, pre-treatment of the NaCl-treated safflower plants with naringenin showed an increase in GR activity, suggesting that under salinity stress naringenin through the binding with the free radicals leading to reduced production of the GR enzyme. We found that by pretreatment of safflower seedlings with naringenin, the NaCl-stimulated H_2O_2 contents were significantly reduced, indicating that naringenin implicated in the activation of antioxidant enzymes involved in the scavenging of ROS. Mekawy et al. (2018) reported that apigenin application could alleviate salinity-induced oxidative damage on rice seedlings by triggering the stimulation of activities of the antioxidant enzymes (CAT) and (APX). In accordance with our findings, Yildiztugay et al. (2020) observed that in response to NaCl, the exogenously applied naringenin increased the activities of SOD, APX and GR in *P. vulgaris*.

It has been shown that under salinity stress a disruption of Na^+ and K^+ homeostasis in cells followed by ion toxicity, adversely influences some major processes such as growth, photosynthesis, and development (Deinlein et al. 2014). Therefore, in plants maintaining cellular homeostasis of Na^+ and K^+ is crucial (Cuin et al. 2011) in many plants salinity tolerance is correlated with a low concentration of Na^+ shoots (Zheng et al., 2009, Li et al., 2012, Mekawy et al., 2018). Our results showed that naringenin pre-treatment of safflower seedlings reduced salinity-induced Na^+ accumulations in roots and shoots (Table 1). Salinity induced a reduction of K^+ content of roots and shoots of safflower seedlings, however in the naringenin pre-treated seedlings significantly compensated the negative impacts of salinity and enhanced K^+ content in roots, suggesting that naringenin may involve in or regulate selective ion uptake in safflower seedlings. Similarly, Mekawy et al. (2018) suggested that apigenin pre-treatment of rice seedlings decreased the extent of Na^+ accumulation and maintained higher K^+ levels under salinity stress.

5. Conclusion

One of the important applications of phenolic compounds is their use as allelochemical compounds. Allelochemicals, which contain a wide range of secondary compounds, have been reported to have a positive effect on their growth and development at the cellular and molecular levels in interaction with plants. Recently, they have been used as an alternative to herbicides and biofertilizers. It has been suggested that phenolic compounds can affect plant growth and physiology and can therefore be used as effective stimulants of plant growth and as an alternative to biofertilizers for their maximum use in field conditions. This research studied the effects of pre-treatment of naringenin on safflower seedlings under salinity stress. The results obtained in the present study showed that naringenin-pretreated safflower seedlings developed apparently better than the untreated seedlings under both the unstressed and NaCl-stress conditions, with respect to increasing plant biomass, enzymatic and non-enzymatic antioxidant, accumulation of osmotic metabolites and K^+/Na^+ ratio as well as lowering the H_2O_2 and MDA contents in the leaves. This result might indicate the involvement of naringenin in protecting plants against salinity-induced oxidative damage. This role could be ascribed to the ability of naringenin to activation an antioxidant defense system by reducing ROS and increasing antioxidant levels and keeping cellular membrane integrity. Our findings confirm the assertion of naringenin being a promising allelochemical that can be used to manage stress, alleviate oxidative conditions associated with salinity complications.

Abbreviations

APX, Ascorbate peroxidase; CAT, Catalase; GR, Glutathione reductase; MDA, Malondialdehyde; RSA, Radical Scavenging Activity; ROS, Reactive oxygen species; SOD, Superoxide dismutase.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Agree.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the experiments: Leila Shabani, Sadegh Farhadian. Performed the experiments: Shahab Hatamipoor. Analyzed the data: Shahab Hatamipoor, Leila Shabani. Wrote the paper: Leila Shabani. Edited the manuscript: Leila Shabani, Sadegh Farhadian.

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Figures

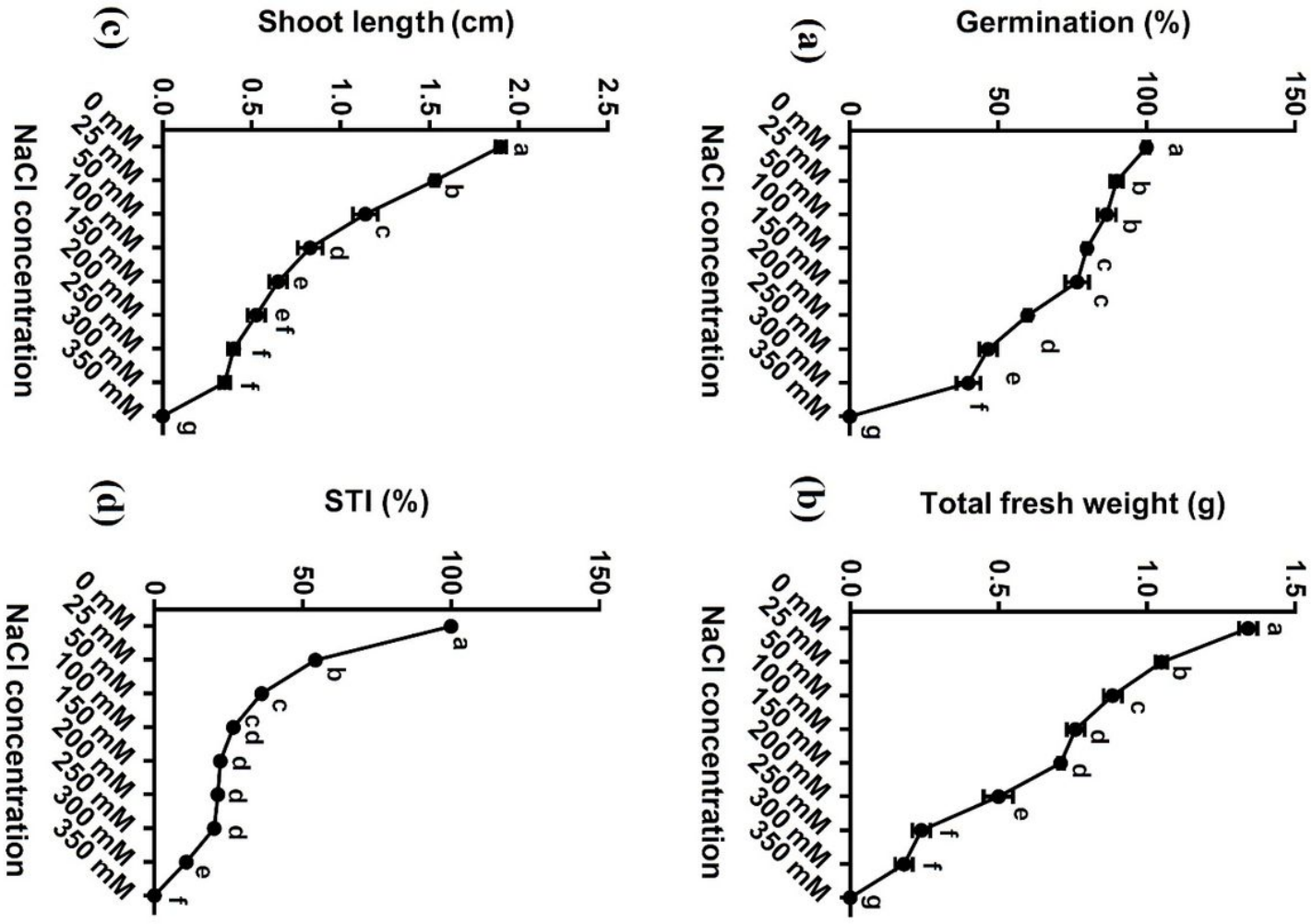

Figure 1

The effects of various concentrations of NaCl (0, 25, 50, 100, 150, 200, 250, 300 and 350 mM) on germination percentage (a), total fresh weight (b) shoot length (c) and salt tolerance index (STI) (d) in the seedlings of safflower. Different letters show significant ($P < 0.05$) differences as determined by l.s.d. test, $n = 3$.

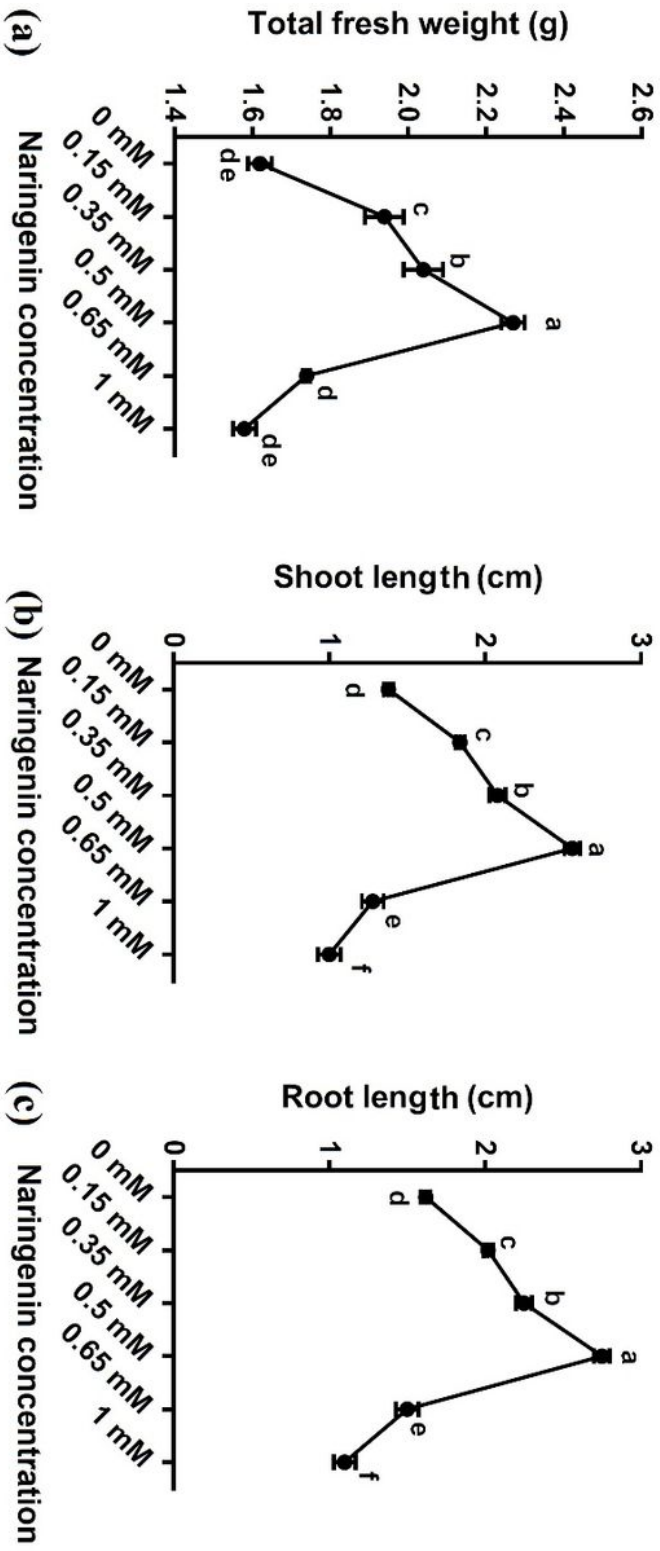

Figure 2

The effects of various concentrations of naringenin (0.15, 0.35, 0.5, 0.65 and 1 mM) on total fresh weight (a), shoot length (b) and root length (c) in the seedlings of safflower. Different letters show significant ($P < 0.05$) differences as determined by l.s.d. test, n = 3.

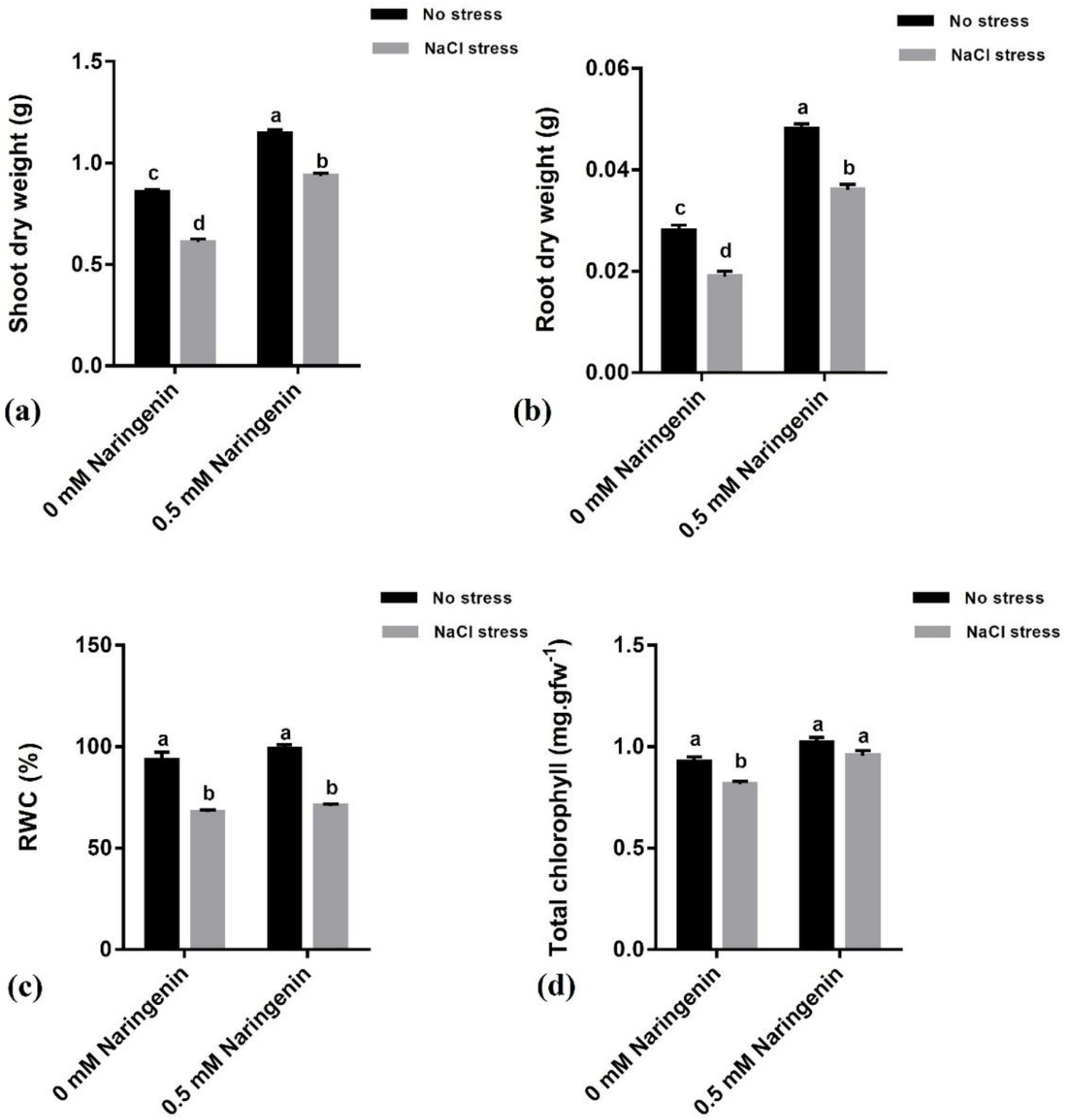

Figure 3

Effect of salinity (25 mM NaCl), naringenin pretreatment and naringenin+NaCl on the dry weight of shoots (a) and roots (b), RWC (C) and total chlorophyll content (D) of safflower seedlings. Different letters show significant ($P < 0.05$) differences as determined by l.s.d. test, n = 3.

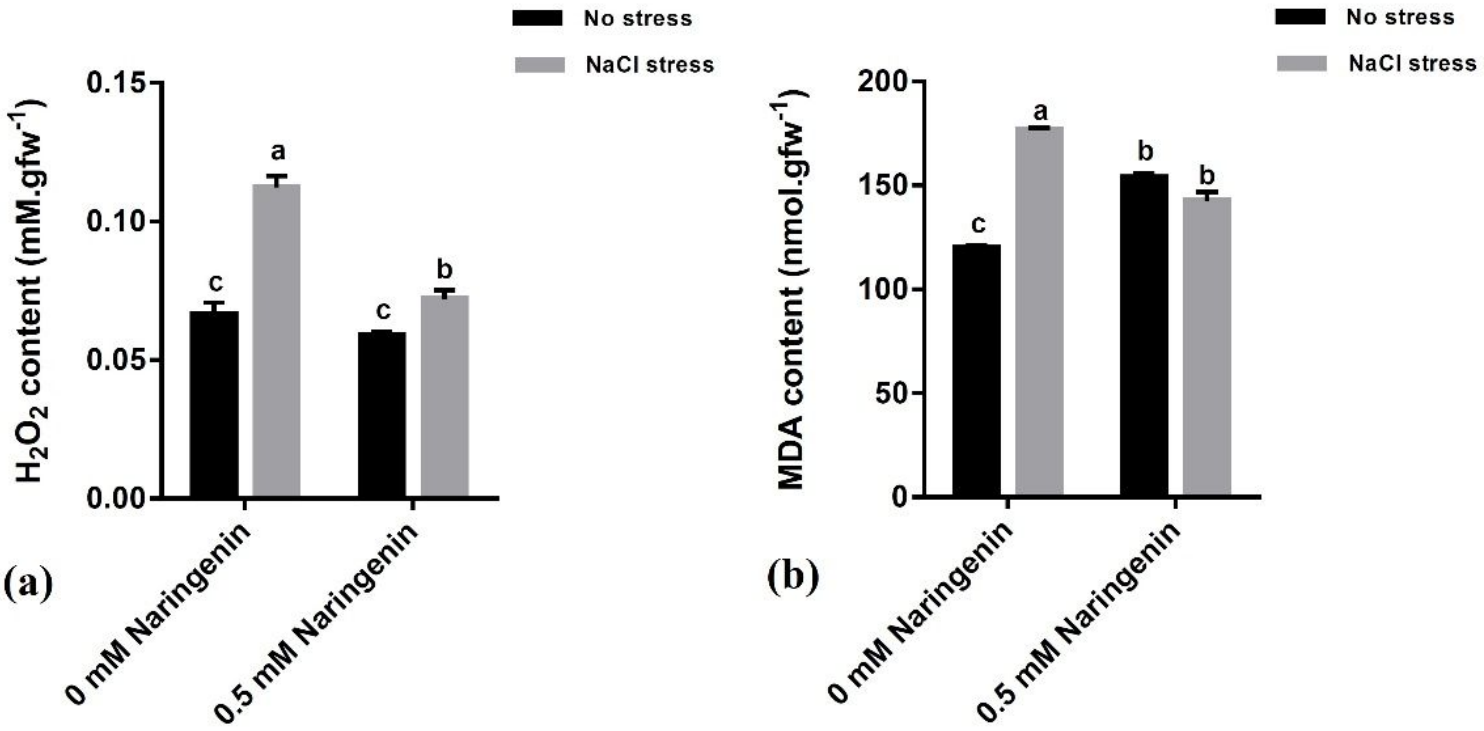

Figure 4

Effect of salinity (25 mM NaCl), naringenin pretreatment and naringenin+NaCl on the (a) H2O2, and (b) MDA concentrations of safflower seedlings. Data represent the mean ± SE (n = 3). Different letters show significant ($P < 0.05$) differences as determined by l.s.d. test, n = 3.

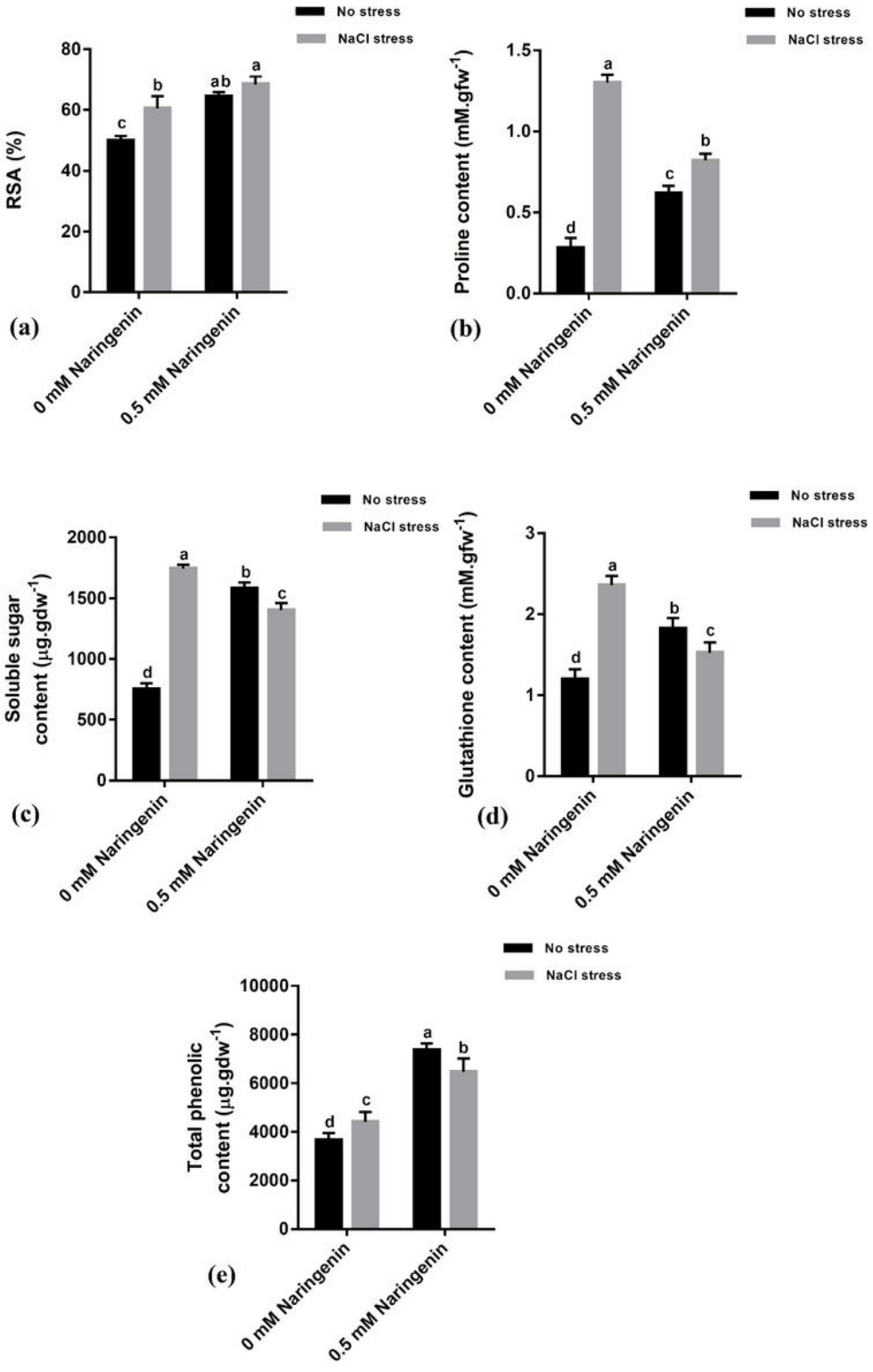

Figure 5

Effect of salinity (25 mM NaCl), naringenin pretreatment and naringenin+NaCl on the (a) RSA (radical scavenging activity), (b) proline (c) total phenolic content, (d) glutathione and (e) soluble sugar content of safflower seedlings. Different letters show significant ($P < 0.05$) differences as determined by l.s.d. test, n = 3.

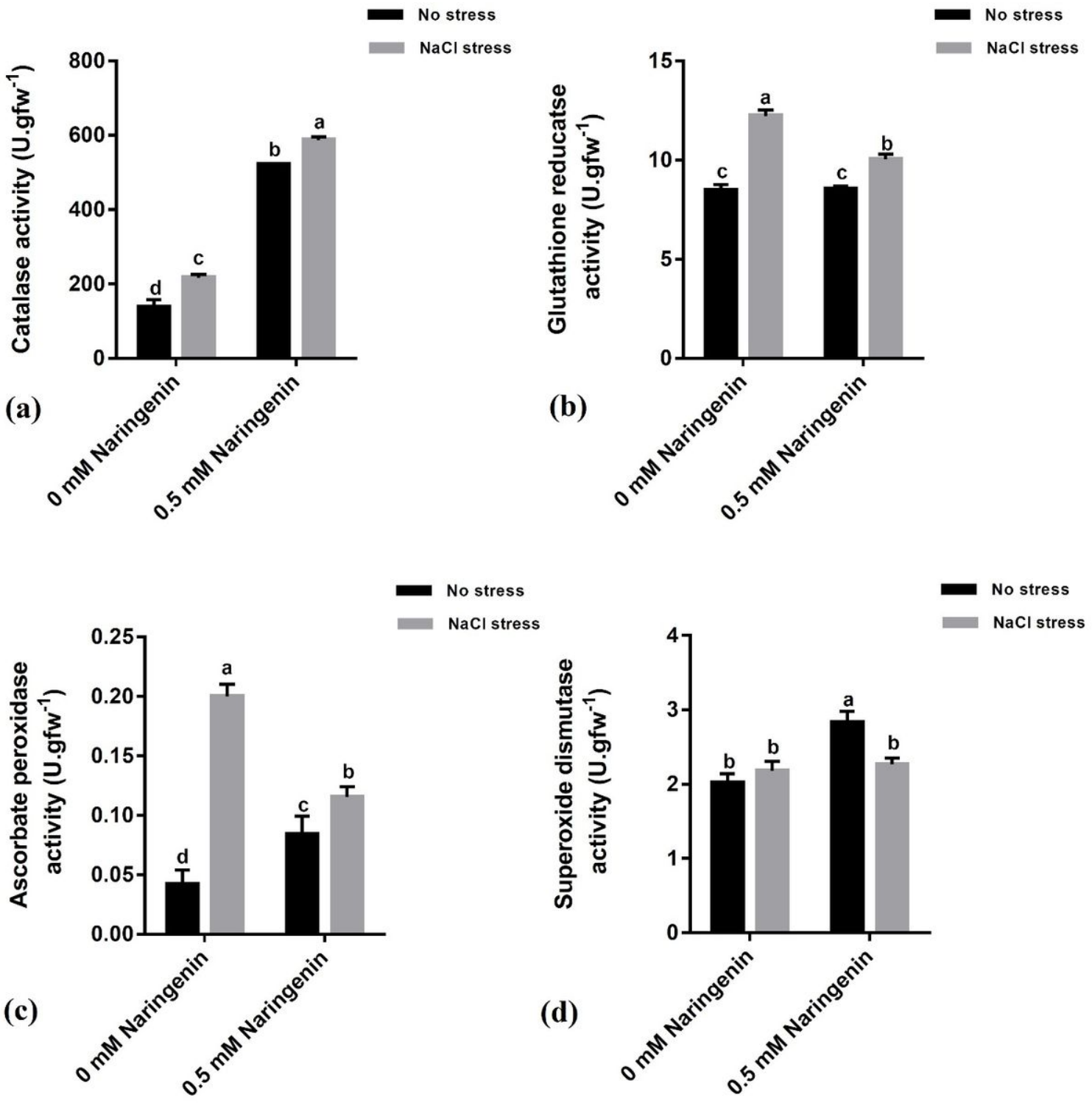

Figure 6

Effect of salinity (25 mM NaCl), naringenin pretreatment and naringenin+NaCl on the (a) CAT, (b) GR, (C) APX and (D) SOD activities of safflower seedlings. Different letters show significant ($P < 0.05$) differences as determined by l.s.d. test, n = 3.