

Alterations in leaf anatomy, quality, and quantity of flavonols and photosynthetic pigments in *Nigella sativa* L. subjected to drought and salinity stresses

Shiva AGHAJANZADEH-GHESHLAGHI, Maryam PEYVANDI*, Ahmad MAJD, Hossein ABBASPOUR

Islamic Azad University, Tehran North Branch, Faculty of Biological Science, Department of Biology, Tehran, Iran;
shiva.aghajanzadeh@daryansalamat.com; m_peyvandi@iau-tnb.ac.ir (*corresponding author); ahmad_majd2005@yahoo.com;
abbaspour75@yahoo.com

Abstract

Nigella sativa was widely used for nutritional and medicinal purposes. The present study investigated the effect of drought and salinity stresses on anatomical leaves structure and some biochemical properties to increase the secondary metabolites. For salt stress plants were treated with NaCl (30, 60 mM), and for drought stress plants were irrigated daily (control), once every two days (2DI) and once every three days (3DI). Compared to control plants, 2DI, 3DI, and NaCl 60 mM treatments increase significantly leaf rutin content, while the amount of rutin in seeds of NaCl 60 mM treated plants showed a significant decrease. 3DI treatment also significantly increased rutin content in seeds compared to NaCl 30 mM and control plants. The maximum level of quercetin ($0.58 \text{ mg g}^{-1} \text{ DW}$), kaempferol ($0.16 \text{ mg g}^{-1} \text{ DW}$), and myricetin ($0.04 \text{ mg g}^{-1} \text{ DW}$) in leaves were gained in both NaCl treatments. However, the flavonol components were affected more at salinity conditions rather than drought. In all treated plants, the amount of these compounds in leaves was more than in seeds. The highest amount of total phenol ($130 \text{ mg g}^{-1} \text{ DW}$), flavonoids ($11.4 \text{ mg g}^{-1} \text{ DW}$), and carotenoid content ($1.55 \text{ mg g}^{-1} \text{ DW}$) of leaves were observed under 2DI stress. Treated plants probably encountered different changes in the anatomical structure of leaves, including the decrease of phloem area, reducing vascular bundles and diameters, decreasing the number, and increasing the volume of cortex cells. The study also corroborates the cooperation between increasing the antioxidant capacity with the total flavonoid, rutin, and quercetin. Results indicated a higher sensitivity of *N. sativa* to drought stress than salinity stress and indicated that moderate salinity and drought could enhance secondary metabolites of seeds in this plant. The formation of potent antioxidants via the treatments could be worthy for pharmaceutical industries.

Keywords: B-carotene; black cumin; flavonols; HPLC; leaf anatomy

Introduction

Before using chemical drugs in the medical care industry, people relied on traditional medicine. World Health Organization has identified medicinal plants as reliable medicinal properties for therapeutic activities (Singh, 2015).

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As an aromatic and medicinal plant, *Nigella sativa* Linnaeus (1753) (black cumin, Ranunculaceae family) has various pharmacological benefits due to its enrichment in essential oil and phenolic compounds in the roots, shoots, and seeds. This worthy plant has antidiabetic, antihistaminic, anti-hypertensive, anti-inflammatory, anti-microbial, antitumor, galactagogue features (Bourgou *et al.*, 2008).

Flavonoids and carotenoids are among the secondary metabolites which play essential roles in plant growth and response to stress.

As important abiotic parameters, salt and drought stress induce osmotic stress, ions toxicity, interruption in the stomatal system, and photosynthesis process, limiting plant growth and productivity, particularly in arid and semi-arid areas (Munns and Tester, 2008).

Moreover, osmotic stress leads to changes in different plants' anatomical, morphological, physiological, and biochemical reactions. In general, these changes involved in the accumulation of osmolytes, reduced photosynthesis, stomatal closure, the induction of stress-responsive genes, the inhibition of cell elongation, disturbances in water and ion uptake, translocation of assimilates, and changes of various metabolic processes, as salt overly sensitive (SOS) pathway, kinases, phosphatases, abscisic acid (ABA), ion transporters and transcription factors (Marcek *et al.*, 2019; Cosic *et al.*, 2020). The effect of salt on plant growth does not occur directly and is usually mediated by effects on cell turgor, photosynthesis, and activation of specific enzymes. As a result, plant growth is impaired in two different osmotic and ionic phases: In the osmotic phase, decreased growth occurs because of the water deficiency caused by the increase in salt content; while in the ionic phase, the accumulation of salt ions in the cell causes cellular destruction (Munns and Tester, 2008; Duarte *et al.*, 2013).

Most of the abiotic stress can be attributed to produce reactive oxygen species (ROS) (Chavarria and dos Santos, 2012). Regarding ROS, the damage degree of macromolecules (DNA, lipid, and proteins), chlorophyll, and other chemical constituents are highly related to the balance between ROS production and its removal by the antioxidant scavenging system (Chavarria and dos Santos, 2012). The antioxidant enzyme systems and nonenzymatic antioxidants could reduce the cytotoxic effects of ROS (Chavarria and dos Santos, 2012). Oxidative stress due to ROS generation leads to the enhancement of flavonoid biosynthesis which has a critical protective role against osmotic stress. Indeed, the disturbance of metabolic processes contributes to increasing phenols, flavonoids, and total antioxidant activity (Wei *et al.*, 2013; Nakabayashi *et al.*, 2014). Phenolic compounds having a pivotal role in scavenging free radicals exist in all parts of plants, including vegetative and reproductive organs (Ksouri *et al.*, 2007; Mahajan *et al.*, 2011; Han *et al.*, 2012). There are few but exciting studies investigating the details of drought and salinity stress on the flavonoid metabolism in higher plants.

As a large class of isoprenoid molecules, carotenoids are an essential part of plant defence system and are divided into hydrocarbon carotenes (e.g., lycopene and β -carotene) and xanthophylls (e.g., lutein). β -carotene, in addition to being involved in photosystems I and II, reduces singlet oxygen production by direct quenching of triplet chlorophyll (Nolan *et al.*, 2017). Photosynthetic pigments are essential to plants, mainly for harvesting light and the production of reducing powers via cyclic photophosphorylation (Farooq *et al.*, 2009). Water stress (Farooq *et al.*, 2009) and osmotic stress (Kiani *et al.*, 2008) cause a decrease in the number of chlorophylls and carotenoids and a change in the ratio of chlorophyll a to chlorophyll b.

A literature review has not disclosed any previous research comparing salt and drought stress on phenol, flavonoid, antioxidant activity, and flavonols components of this plant. Efforts conducted to explain salinity or drought stress on secondary metabolites of this plant are very limited. However, different researchers studied the effect of salinity on fatty acids, phenolics, and antioxidant activity of this medicinal plant (Bourgou *et al.*, 2010, 2012; Bensalem *et al.*, 2020; Golkar *et al.*, 2020; Iqbal *et al.*, 2021). The impact of drought stress on phytochemical components was investigated by several studies (Bayati *et al.*, 2020; Ghamarnia and Jalili, 2013; Mozzafari *et al.*, 2000). Mozzafari *et al.* (2000) indicated the enhancement of seed oil in *N. sativa* due to reduced irrigation supported by Haj Seyed Hadi *et al.* (2012).

Bayati *et al.* (2020) indicated the effects of irrigation regimes on enhanced yields in black cumin. Furthermore, it showed that black cumin genotypes were both qualitatively and quantitatively affected by drought level.

Some literature indicated moderate tolerance to drought stress in *N. sativa* (Ghamarnia and Jalili, 2013; Mozzafari *et al.*, 2000).

To the best of our knowledge, no study has been performed concerning the changes of flavonoid and carotenoid components in *N. sativa* in response to abiotic stress. This research aimed to identify the effects of salinity and drought stress on the phenolic and flavonoid content, photosynthetic pigments, antioxidant activity, and leaf anatomy of this worthy medicinal plant. Moreover, we conducted this research to find out does black cumin tolerates salinity and drought stress? The amount of valuable secondary metabolites in response to these conditions was discussed.

Materials and Methods

Plant material, growth, and treatment conditions

Nigella sativa seeds were prepared from Pakan Company (Esfahan Province, Iran). Sterilized seeds were planted directly in plastic pots (15x12 cm) that were placed in greenhouse conditions. Pots filled with sterilized coco peat: perlite (1:1) mixture. Watering was performed with tap water every day and with half strength of Hoagland solution (pH6.5) twice a week. After 30 days, the treatments were initiated when seedlings had two to three leaves. Three plants were let to grow in each pot. Six replicate pots were kept for each treatment. The pots were arranged in a complete randomized design. Plants were subjected to salinity stress by adding two NaCl levels (30 and 60 mM) to half strength of Hoagland solution. For drought treatments, watering was performed daily (control), once every two days (2DI), and once every three days (3DI). The daily amount of water and Hoagland solutions added to the pots were the same for all treatments.

Four weeks after the beginning of treatments, anatomical structure and chemical composition were analyzed.

All experiments were performed in greenhouse conditions with 60-70% humidity, and average day/night temperatures were 28 °C/22 °C.

Determination of photosynthetic pigments

The absorbance of the acetone leaf extracts was read at 663, 645, and 470 nm using a UV-visible spectrophotometer (Philler scientific su-6100, USA). To measure the chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophylls, and carotenoids. The content of photosynthetic pigments was calculated following the equations of Lichtenthaler (1987).

$$\text{Chl a (mg l}^{-1}\text{)} = 12.25 A_{663} - 2.79 A_{645}$$

$$\text{Chl b (mg l}^{-1}\text{)} = 21.5 A_{645} - 5.10 A_{663}$$

$$\text{Total Chl} = \text{Chl a} + \text{Chl b}$$

$$\text{Total carotenoids (mg l}^{-1}\text{)} = [(1000 A_{470} - 1.82 \text{Chl a} - 85.02 \text{Chl b})/198]$$

β-carotene extraction and estimation

Four g of leaf powder was mixed with 0.3 g MgCO₃. The mixture was ground with 25 ml of cold acetone. The extract was filtered using a Whatman filter paper. Fifteen ml hexane and 10 ml extract were mixed. Then allowed to stand for 20 minutes. The mixture was then washed with 30 ml distilled water, and the lower aqueous phase was discarded. The washing with water was repeated thrice. 4.5 ml acetone was added to the upper layer and then diluted to volume (20 ml) with hexane (Ball, 1988). Absorbance was read at 453 nm. The following formula measured the β-carotene content:

$$\beta\text{-carotene } (\mu\text{g g}^{-1} \text{ DW}) = \frac{A \times V \text{ (ml)} \times 10^4}{E_{1\text{ cm}}^{1\%} \times W \text{ (g)}}$$

Where A = Absorbance at 453 nm; V = Total extract volume(ml); W = sample weight(g); $E_{1\text{ cm}}^{1\%} = 2592$ (β -carotene Extinction Coefficient in hexane).

Phenolic compound extraction

For total phenols and flavonoids assay, dried powder of leaves and seeds (0.5 g) were homogenized with 5ml of methanol 80% and shaken for 24h. Solutions were then centrifuged at 13000×g for 15 min, and the supernatants were used for the determination of total phenols, flavonoids and flavonols assay.

Determination of total phenol content

The total phenolic content was determined by using Folin-Ciocalteu's assay (Marinova *et al.*, 2005). Seeds and leaves phenolic extract (2.5 ml) was mixed with distilled water (2.5 ml) and 15 times diluted Folin (0.2). Five minutes later, 7% sodium carbonate (Na_2CO_3) (3ml) was added. After 90 minutes the absorbance was determined at 750 nm.

As standard curves, solutions of gallic acid (Sigma, Germany) ($0\text{--}500 \mu\text{g ml}^{-1}$) were used ($R^2 = 0.997$) that expressed as mg GAE) g^{-1} leaves and seeds dry weight.

Determination of total flavonoid contents

The total flavonoid content was determined using the aluminum chloride colorimetric method with some modification (Chang *et al.*, 2002). Seeds and leaves phenolic extracts (0.5 ml) was mixed with ethanol 95% (1.5ml), 10% aluminium chloride (0.1 ml), potassium acetate 1M (0.10 ml) and distilled water (2.80 ml). After 30 minutes the absorption was read at 415 nm. Quercetin (Sigma, Germany) solutions with concentrations ($0\text{--}100 \mu\text{g ml}^{-1}$) were used for standard curve. Flavonoids content was expressed mg Quercetin. g^{-1} DW.

Measurement of flavonols by high-performance liquid chromatography (HPLC)

0.5 g of dried leaves were hydrolysed with 5 mL of methanol 80% and shaken for 24h, then centrifuged at 13500×g for 15 min and the supernatants were topped up to 5mL by methanol 80%. The extracts were filtered via $0.45 \mu\text{m}$ nylon filters and stored at $4 \text{ }^\circ\text{C}$ for further analysis.

Quercetin, kaempferol, myricetin, and rutin standards were provided by Sigma (USA) and prepared in 80% methanol. For each compound, the calibration curves were drawn using different concentrations of standards.

The analysis of Quercetin, kaempferol, myricetin, and rutin was done using Agilent 1260 infinity ii HPLC system (Agilent, USA), covering Binary Pump, Vial sampler with integrated column compartment, 6.0 μL heater and sample cooler, Diode Array Detector WR with a standard 10-mm flow cell, 1100 Autosampler, 1100 Thermostatted Column Compartment, 1100 Diode Array Detector with a standard 10-mm flow cell, and Agilent OpenLAB CDS Version 2.1. The separation of compounds was carried out using Agilent ZORBAX Eclipse Plus C18, $4.6 \times 150 \text{ mm}$, $5 \mu\text{m}$. The used mobile phase was methanol: 200, acetonitrile: 100, acetic acid: 10, phosphoric acid: 10, and 200 mL of water. The injection volume was 20 μL by a flow rate of 0.6 ml/min. The integrated peak areas of the sample against the standard curves were used to quantify compounds in terms of mg ml^{-1} (Azarafshan *et al.*, 2020).

Antioxidant activity

The DPPH assay was determined according to the Akowuah *et al.* (2005) method. 2, 2-diphenyl-1-picrylhydrazyl (DPPH, 0.004 %) was prepared freshly before analysis. Five volumes (50-250 μl) of the seeds

and leaves phenolic extract were added to 1 ml of DPPH and incubated for 30 min. The absorbance was read at 517 nm, and the inhibition percentage was calculated with $I\% = ((A_b - A_s)/A_b) \times 100$ where A_b is the absorbance of control (the reaction solution without seed or leaf phenolic extract) and A_s is the absorbance of samples (the reaction solution with 50-250 μ l of seed or leaf phenolic extract). The percentage of inhibition after 30 min was plotted against concentration, and the equation for the line was used to obtain the IC50 (half maximal inhibitory concentration) values.

Leaf anatomy

The shoots and roots of plants were separated after harvesting. Leaf parts were cut and washed with water to remove different pollution. The leaves were placed in 70 percent ethanol solution for 15 days. After that, the sections were examined under a microscope, and consequently, microphotography was done. A double staining dehydration procedure (safranin and fast green) was used to prepare permanent slides to study the anatomy changes of leaves (Johansen, 1940). Measurements and micrographs were made using a digital camera (Nikon FDX-35) equipped with a Nikon stereo-microscope (Nikon 104, Japan). The anatomical parameters observed in this present study were: the thickness of xylem and phloem cells, xylem area, phloem area, cortex area, the area of upper and lower epidermis, cuticle thickness, the thickness of the upper and lower epidermis, and the area of the vascular system. All measurements were performed by image tools software (Wilcox *et al.*, 2002).

Statistical analysis

Three replications of each treatment were used for biochemical analysis. All data were statistically analysed using the software SPSS (ver.22). Data were indicated as means \pm standard error (SE). One-way analysis of variances (ANOVA) was used to compare the means and determine the impact of the factors (drought and salt stresses) on the variables studied. Differences between treatments were tested by Duncan's multiple range tests at a significance level of $P \leq 0.05$.

Results

Leaf anatomy

Comparing leaves characteristics under drought and salinity stresses to control by 'Duncan's Multiple Range Test demonstrated:

Cuticle thickness: Drought stress had no significant effect on cuticle thickness compared to control plants. Conversely, NaCl 60 mM stress decreases it very significantly (70% reduction).

Upper and lower epidermal cell thickness: Upper epidermal cell thickness did not show any significant difference under drought and salinity stresses to control. While, lower epidermal cell thickness showed significantly decreased when subjected to 3DI (40% reduction) and under salinity stresses of NaCl 30mM and 60mM (about 27% and 43% reduction, respectively) (Table 1).

Xylem and Phloem thickness: The drought and salinity treatments did not significantly affect the xylem cell's thickness. In contrast, the phloem thickness in plants under salinity stress of 30mM and 60mM NaCl showed a decrease of about 29% and 43% reduction, respectively.

Xylem and phloem area: Both parameters showed a significant decrease under salinity and drought stresses (about 30 to 50% reduction).

Upper and lower epidermal cell area: the upper epidermal cell area was also significantly decreased by salt treatments, and any significant difference was not observed under drought treatments. Moreover, all drought and salt treatments decreased the lower epidermal cell area significantly (about 27 to 50% reduction) (Table 1).

Cortex and vascular bundles area: The cortex cell area of leaf increased significantly under all drought and salt treatments except 2DI. The highest percentage increase was observed in plants under salinity stress (about 400% increase).

The vascular bundle area decreased significantly under all drought and salinity treatments (Figure 1, Table 1). The highest decrease was observed in 3DI and NaCl 30 mM (58% and 54% reduction, respectively).

Moreover, Figure 1 indicated some structural changes in leaves under abiotic stress including the decrease of lower epidermis thickness, the decrease of xylem and phloem area, decreasing the number of cortex cells, and increasing the volume of them that ultimately lead to increase of the area.

Table 1. The effect of drought and salinity on leaf-anatomical features of *N. sativa* L

Treatment	Cut. thick. (μm)	Uep. thick. (μm)	Lep. thick. (μm)	Xyl. thick. (μm)	Phl. thick. (μm)	Xyl. area (μm^2)	Phl. area (μm^2)	Cor. area (μm^2)	Uep. area (μm^2)	Lep. area (μm^2)	Vas. area (μm^2)
Control	6.24 \pm 0.89ab	24.25 \pm 1.1a	24.76 \pm 0.88a	14.81 \pm 0.71a	4.57 \pm 0.46b	25634.66 \pm 2654.22a	7184.58 \pm 291.98a	2870.03 \pm 373.99c	627.09 \pm 47.23a	588.71 \pm 63.04a	53793.95 \pm 5166.48a
2DI	8.19 \pm 0.64a	20.69 \pm 1.44a	23.57 \pm 1.56a	15.07 \pm 0.74a	6.61 \pm 0.49a	18038.21 \pm 2575.29b	3937.36 \pm 487.88b	4672.14 \pm 779.72bc	441.35 \pm 68ab	427.78 \pm 103.55b	34413.67 \pm 3648.17b
3DI	5.59 \pm 0.95b	24.27 \pm 3.59a	14.93 \pm 0.91c	13.25 \pm 1.44a	3.77 \pm 0.31bc	16957.63 \pm 2580.07bc	3565.15 \pm 574.78b	13797.72 \pm 753.02a	491.74 \pm 119.16ab	301.33 \pm 21.03c	22697.08 \pm 1930.18c
NaCl 30 mM	6.88 \pm 0.55 ab	22.32 \pm 0.89a	18.05 \pm 0.47b	13.27 \pm 0.62a	3.23 \pm 0.39c	10560.05 \pm 703.11c	4358.06 \pm 746.4b	5624.22 \pm 281.62b	290.13 \pm 28.9b	269.23 \pm 19.35c	24579.94 \pm 1332.56c
NaCl 60 mM	1.87 \pm 0.22c	20.21 \pm 1.44a	13.88 \pm 1.05c	14.21 \pm 0.89a	2.58 \pm 0.42c	21375.96 \pm 2413.11ab	2851.82 \pm 224.34b	15078.33 \pm 939.56a	315.75 \pm 31.54b	349.44 \pm 34.25bc	39704 \pm 2841.79b

Values (means of three replicates \pm SE) of each parameter followed by at least one same letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test, Control: daily watering, 2DI: once every two days irrigation, 3DI: once every three days irrigation.

NaCl 30 mM and NaCl 60 mM: Salt stress levels.

Abbreviation: Cut. thick: cuticle thickness; Uep. thick: upper epiderm thickness; Lep. thick: lower epiderm thickness; Xyl. thick: Xylem cell thickness; Phl. thick: Phloem cell thickness; Xyl. area: Xylem area; Phl. area: Phloem area; Cor. area: Cortex area; Uepi. area: upper epiderm area; Lep. Area: Lower epiderm area; Vas. area: Vascular area

Photosynthetic pigments

A significant decrease in the content of Chl a, b, and total Chl was observed under all drought and salt treatments (Table 2). The minimum level of chl a (4.34 mg l^{-1}), chl b (2.64 mg l^{-1}) and total chl (7.26 mg l^{-1}) were gained in NaCl 60 mM. The amount of total chlorophyll in different treatments compared to the control showed a decrease of about 45 to 50%. Total carotenoid content increased significantly under 2DI and NaCl 30 mM treatment. In contrast, significant increase in β -carotene in response to 3DI (40% increase) and very significantly increase under NaCl 60 mM treatment (130% increase). (Table 2).

Phenolic compounds

Total phenols content (Leaf and seed)

The total phenol values of the extract solutions reacted with Folin–Ciocalteu reagent compared with the Gallic acid standard solutions are shown in Table 3.

According to the findings in table3, the mild drought treatment (2DI) substantially increased the total phenol content of both leaves and seeds. In contrast, the severe drought conditions (3DI) did not affect this parameter (Table 3). The total phenolic content varied in the different treatments ranging from 83.72 mg g^{-1} DW to 158.31 mg g^{-1} DW, both detected in the seeds. The highest amount of total phenolic was observed in salt stress at NaCl 60- and 30-mM treatment. In contrast, 3DI treatment showed the lowest amount of total phenolic content in leaves samples.

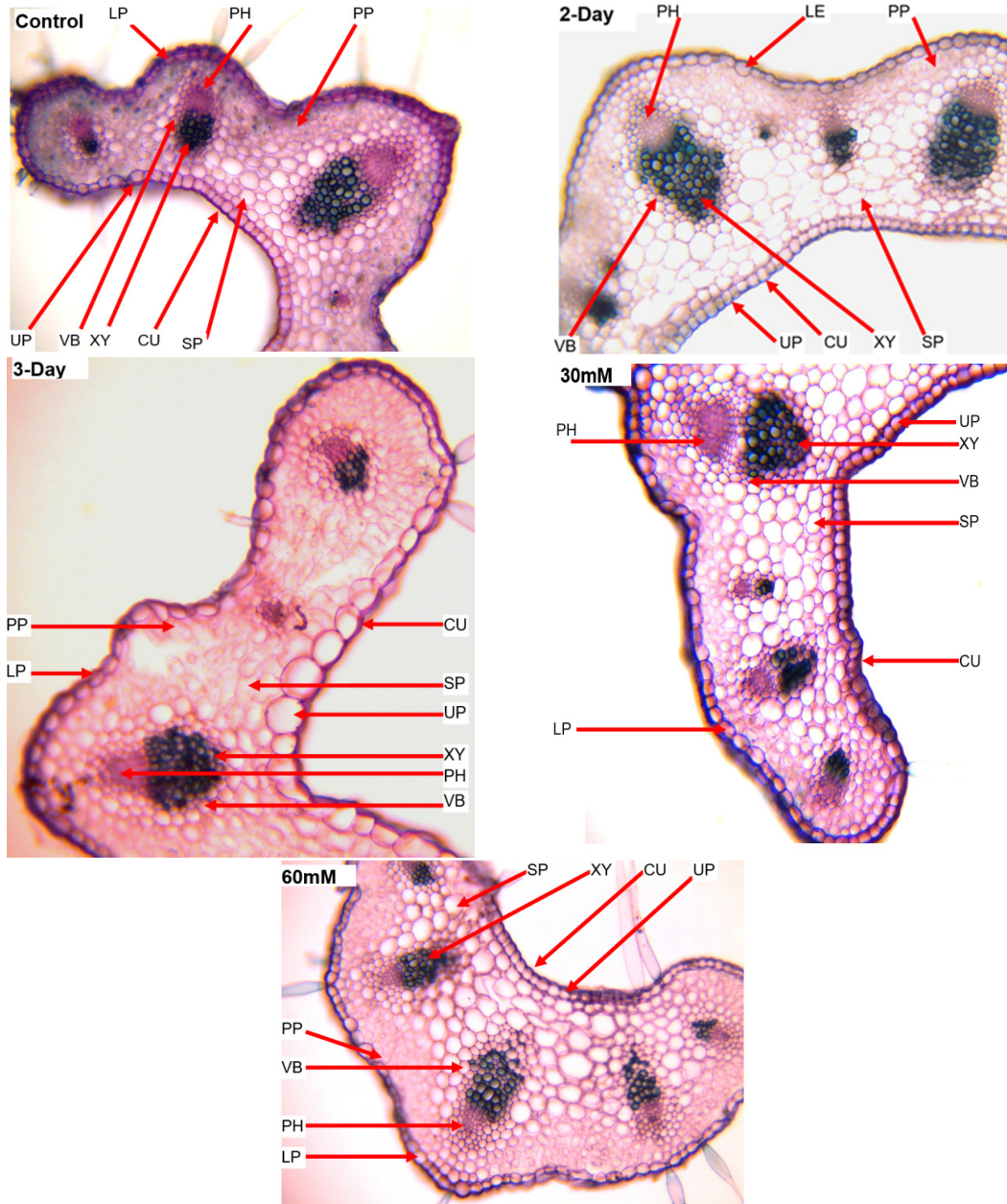


Figure 1. The anatomical structure of leaves under the different treatments of drought and salinity stresses. Control: daily watering, 2-day: Once every two days irrigation, 3-day: Once every three days irrigation; 30mM: salinity with NaCl 30mM; 60mM: salinity with NaCl 60mM (10 xs). VB: vascular bundle; PP: palisade parenchyma; SP: spongy parenchyma; UP: upper epidermis; LP: lower epidermis; XY: xylem; PH: phloem; CU: cuticle.

Table 2. The effect of drought and salinity on photosynthetic pigments components of *N. sativa* L.

Treatment	Chla (mg l ⁻¹)	Chlb (mg l ⁻¹)	Total Chl (mg l ⁻¹)	Carotenoid (mg l ⁻¹)	β-carotene (μg g ⁻¹ DW)
Control	8.68±0.39a	6.62±0.5a	15.3±0.66a	0.84±0.06b	20.51±2.29c
2DI	5±0.37b	2.57±0.41b	7.56±0.23b	1.55±0.23a	18±2.09c
3DI	4.5±0.45b	2.65±0.47b	7.27±0.18b	1.35±0.19ab	28.63±4.41b
NaCl 30mM	4.57±0.29b	3.80±0.26b	8.35±0.34b	1.51±0.15a	20.32±1.43c
NaCl 60mM	4.34±0.17b	2.64±0.12b	7.26±0.22b	1.29±0.14ab	47.33±0.67a

Values (means of three replicates ± SE) of each parameter followed by at least one same letter are not significantly different at P≤0.05 based on Duncan's multiple range test, Control: daily watering, 2DI: Once every two days irrigation, 3DI: Once every three days irrigation, NaCl 30 mM and NaCl 60 mM: Salt stress levels.

The total phenolic content of seeds increased proportionally with the increase in salt concentration (Table 3). At NaCl 60 mM, total phenol was 158.31 mg g⁻¹ DW, which was 81% higher than that of control seeds (87.03 mg g⁻¹ DW), while this ratio was 21% increase at NaCl 30 mM (Table 3). In seed extracts, the results also revealed that salt treatments had more influence on phenolic content increasing than drought treatments.

Except for NaCl-stressed (60 mM) seeds, the total phenols of leaves and seeds under drought and salinity stresses showed that leaves had more total phenol content than seeds.

Significantly, seed extracts of plants showed more value of phenolic content with increasing NaCl concentrations to 30 and 60 mM, respectively, compared to drought treatments and the control.

Table 3. Effect of drought and salt on total phenol, flavonoid content, and antioxidant activity (IC50) in leaves and seeds of *N. sativa*

Treatment	Total Phenol (mg g ⁻¹ DW)		Total flavonoid (mg g ⁻¹ DW)		DPPH scavenging activity IC50 (mg g ⁻¹ DW)	
	leaf	seed	leaf	Seed	Leaf	seed
Control	115± 10.1ab	87.03 ±4.7c	8.96±0.44a	2.12±0.1c	105.8±1.92ab	117.37±3.88a
2DI	130.21±6.22a	97.26±1.71b	11.4±0.46a	2.07±0.02c	95.48±1.10b	88.21±0.35b
3DI	112.91±5.58b	83.72±1.7c	9.33±1.33a	2.29±0.09bc	105.42±5.93ab	90.53±0.31b
NaCl 30 mM	124.93±6.72a	105.69±2.51b	9.66±0.56a	2.45±0.02b	95.6±2.59b	90.21±0.67b
NaCl 60 mM	115±7.09ab	158.31±3.42a	6.26±0.63b	3.05±0.12a	109.45±3.05a	60.87±9.22c

Values (means of three replicates ± SE) of each parameter followed by at least one same letter are not significantly different at P≤0.05 based on Duncan's multiple range test, Control: daily watering, 2DI: Once every two days irrigation, 3DI: Once every three days irrigation, NaCl 30 mM and NaCl 60 mM: Salt stress levels.

Total flavonoid content (Leaf and seed)

Total flavonoids content of seeds and leaves of *Nigella sativa* cultured on different treatments are shown in Table 3. Results indicated salinity and drought stress significantly affected the total flavonoid content of leaves and seeds.

In leaves, extract of 2DI *N. sativa* had the highest total flavonoids (11.4 mg g⁻¹ DW) followed by NaCl 30 mM (9.66 mg g⁻¹ DW). All treatments had more total flavonoid content than control (8.96 mg g⁻¹ DW) except NaCl 60 mM, which showed the lowest total flavonoid content (6.26 mg g⁻¹ DW).

In seeds, extract of NaCl 60 mM stressed plants had the highest total flavonoids (3.05 mg g⁻¹ DW) followed by those treated with NaCl 30 mM (2.45 mg g⁻¹ DW). In all treatments, *N. sativa* seed extracts had more total flavonoid content than control (8.96 mg g⁻¹ DW) except those from the 2DI treatment, which showed the lowest total flavonoid content (2.07 mg g⁻¹ DW).

Flavonols quantification by HPLC analysis in leaves and seeds

The amounts of flavonol compounds in the leaves and seeds of *N. sativa* at different salt and drought stresses are shown in Table 4, which including rutin, myricetin, quercetin, and kaempferol.

Based on these results, more flavonols were observed in leaves than in seeds.

Leaves: Rutin and quercetin are the most abundant components (9.19 and 0.58 mg g⁻¹ DW, respectively) in control and treated plants' leaf extract among the flavonols examined in this study. In contrast, myricetin was found in leaves at lower concentrations (0.012-0.039 mg g⁻¹ DW) than other flavonol components. Though the differences were not significant, the amount of rutin in the leaves differs significantly across all treatments (except NaCl 30 mM), as seen in table 4.

Leaves of salt treated plants produced significantly higher quercetin content, compared to the control and drought treated plants. A high amount of quercetin was observed in the extract of NaCl 30mM stressed leaves, followed by those in the extract of NaCl 60mM, while the lowest amount was obtained in control plants. Salinity stress at NaCl 60 mM had a significant effect on myricetin value and it increased its amount of it (threefold above control one), while drought stresses had no significant influence on this flavonols component. A similar result was observed in the kaempferol value, but the highest values occurred at NaCl 30 mM treatment (Table 4).

Seeds: Rutin and kaempferol had the most value to other flavonols in control and all treatments, while quercetin had the lowest amount of it. Salt and drought treatments influenced the rutin significantly (Table 4) (Figure 2). A high amount of rutin was observed in 3DI and NaCl 30mM, 1.02 and 0.942 mg g⁻¹ DW, respectively. Whereas, at NaCl 60mM treatment lowest amount of rutin was observed (0.391 mg g⁻¹ DW). A similar range of rutin values was obtained from control and 2DI extracts (0.82 mg g⁻¹ DW). Thus, NaCl 60mM treatment had a significant positive effect on rutin amount, and 3DI had a significant negative effect. Also, these results were observed in myricetin quantities. At NaCl 60mM, this component had the lowest amount, while the highest values of myricetin occurred in 3DI treatment. Similar ranges were observed at the control and other treatment without significant differences.

Though the differences were not significant, increasing the seed's quercetin value was observed in NaCl 60 mM treatment.

Salt and drought treatments influenced the seed kaempferol compounds significantly, so that these treatments decreased the quantity of it to the control one. The highest value of myricetin occurred in control plants 0.1 mg g⁻¹ DW, while this amount decreased to 0.04 mg g⁻¹ DW in 3DI. Other treatments showed a similar range of this value (0.042-0.051).

Table 4. HPLC analysis of flavonol compounds in *N. sativa* leaves and seeds under drought and salt treatments

Treatm.	Rutin (mg g ⁻¹ DW)		Myricetin (mg g ⁻¹ DW)		Quercetin (mg g ⁻¹ DW)		Kaempferol (mg g ⁻¹ DW)	
	leaf	seed	leaf	Seed	Leaf	seed	leaf	seed
Control	6.03± 0.83b	0.83± 0.03b	0.013± 0.003b	0.005± 0.001bc	0.12± 0.026c	0.0002± 0.00007b	0.011± 0.004c	0.10± 0.006a
2DI	9.19± 2.08a	0.83± 0.03b	0.012± 0.002b	0.006± 0.001b	0.27± 0.02bc	0.0003± 0.00008b	0.08± 0.023abc	0.05± 0.003bc
3DI	8.00± 0.35a	1.02± 0.04a	0.019± 0.008b	0.032± 0.003a	0.20± 0.00c	0.0002± 0.00005b	0.05± 0.001bc	0.04± 0.004c
NaCl 30 mM	5.78± 0.28b	0.94± 0.03ab	0.027± 0.002ab	0.005± 0.001bc	0.58± 0.17a	0.0002± 0.00004b	0.16± 0.05a	0.05± 0.003b
NaCl 60 mM	8.57± 0.14a	0.39± 0.08c	0.039± 0.009a	0.001± 0.00c	0.54± 0.11ab	0.001± 0.00007a	0.11± 0.016ab	0.04± 0.002bc

Values (means of three replicates ± SE) of each parameter followed by at least one same letter are not significantly different at P≤0.05 as determined by Duncan's multiple range test, Control: daily watering, 2DI: once every two days irrigation, 3DI: once every three days irrigation, NaCl 30 mM and NaCl 60 mM: Salt stress levels

Antioxidant activity (Leaf and seed)

Antioxidant activity was determined by evaluating the effects of leaf and seed extracts on DPPH free radical scavenging activities of leaf and seed extracts produced under in all treatments.

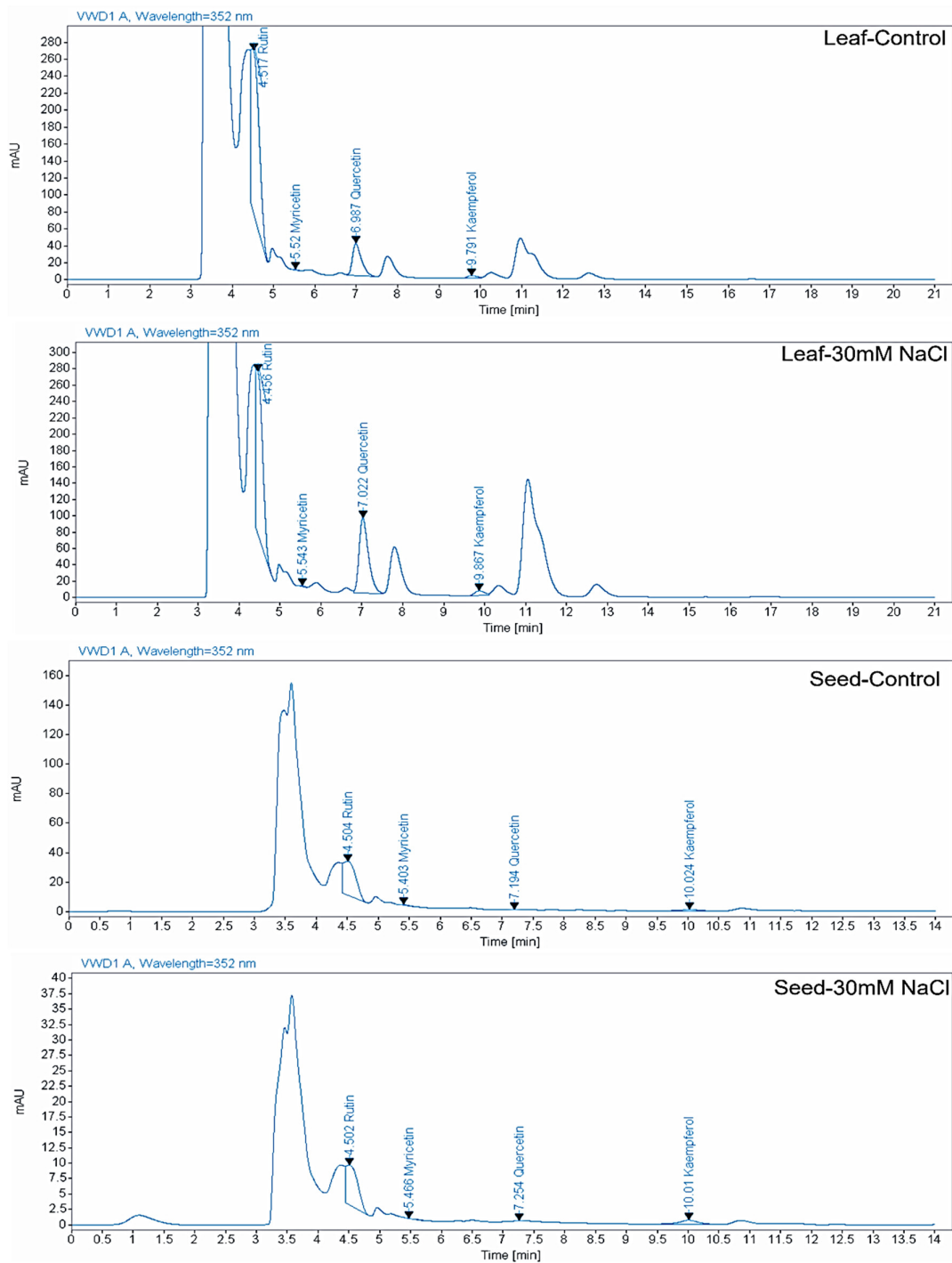


Figure 2. HPLC chromatograms of flavonol compounds in *N. sativa* leaves and seeds (Control and 30 mM NaCl treatment)

Salt and drought treatments significantly affected the leaf and seed's antioxidant activity (Table 3). In leaves methanolic extract, the minimum level of IC₅₀ was observed in 2DI and NaCl 30 mM treatments, which means the maximum level of leaves antioxidant activity belongs to these treatments.

In seeds methanolic extract, the minimum and maximum levels of IC₅₀ were observed in treatments of NaCl 60 mM and control, respectively, which means the maximum and minimum level of seeds antioxidant activity belong to these two treatments, respectively.

Discussion

To reduce water consumption, *N. Sativa* probably encountered different changes in the anatomical structure of leaves such as decreasing the phloem area, reducing vascular bundles and their diameters, decreasing number, and increasing the volume of cortex cells.

Reducing the area of the xylem is likely to play a role to adapt the plant to drought conditions because smaller diameter cells reduce the hydraulic conductivity of xylem, which in turn slows down the movement of water and helps the plant to slow down more quickly and therefore use available water for a more extended period (Talamè *et al.*, 2007). Reducing the number, diameter, and area of the vascular system under abiotic stress were reported in some species such as *Leptochloa fusca*, *Salsola dendrites*, and olive tree (Ola *et al.*, 2012; Abdelkrim *et al.*, 2014; Fayek *et al.*, 2018).

The decrease in chlorophyll content indicates a photoprotection mechanism by reducing light absorbance (Elsheery and Cao, 2008). The parameters such as intensity, duration, phenological phase of growth, and genetic factors could reduce photosynthetic pigments (Kabiri *et al.*, 2014). On the other hand, the stomata closure due to osmotic stress induced by salinity could reduce the photosynthesis assimilation via the limitation of the entry of CO₂ (Acosta-Motos *et al.*, 2017). In this content, Bensalem *et al.* (2020) indicated that stomatal conductance and photosynthetic assimilation were limited in leaves of black cumin treated with NaCl. The carotenoid content of leaves was enhanced only under 2DI and salinity stress (NaCl 30 mM). While any significant difference in terms of carotenoid content was not observed in plants subjected to 3DI and salinity stress (NaCl 60 mM). These results indicated that in *N. Sativa*, carotenoids are involved tolerance to 2DI and salinity stress (NaCl 30 mM). The enhancement of carotenoids had an essential role as a nonenzymatic antioxidant in photosynthesis photoprotection (Habibi and Ajory, 2015). A similar result was recorded in *Nigella sativa* and *Aloe vera* species during 2DI stress. The accumulation of protective pigments such as carotenoids in leaves developed an effective photoprotection mechanism (Habibi, 2018; Kabiri *et al.*, 2014).

Genetic factors and environmental conditions influence phenolic compounds of plants which lead to plant protection against oxidative stress through antioxidants and antiradical (Azarafshan *et al.*, 2020). However, intraspecific variation in phenol contents is a remarkable parameter which needs to be regarded when studying plant tolerance to stresses (Valifard *et al.*, 2014). The observed increase in phenolic content of seeds in this work was possible due to this antioxidative mechanism against oxidative stress induced by salt and drought stress (Han *et al.*, 2012). The tolerance of *N. sativa* to moderate salinity (NaCl 30 mM) and drought (2DI) matched with increasing of total phenol, the flavonoid in seeds while their tolerance to high salinity (NaCl 60 mM), not high drought, coincided with increasing of total phenol and flavonoid in seeds. It is probably that more accumulation of Na⁺ and Cl⁻ in seeds leads to an increase in the polyphenol content in their regard to leaves. Before, an enhancement of their transfers from roots to shoots of *N. sativa* under increasing salinity levels was indicated (Hussain *et al.* 2009). Moreover, the results are in accordance with the stimulation of phenylpropanoid metabolism in response to abiotic stresses (Yaginuma *et al.*, 2002). In a similar study, Bourgou *et al.* (2010) reported the enhancement of phenolic acid and flavonoid composition of *N. sativa* seeds treated with NaCl 60 mM, leading to the enhancement of antioxidant activity in this plant. The enhancement effects of moderate salinity on some valuable volatile oils and total phenolic compounds were observed in some plants like *Salvia mirzayanii* (Valifard *et al.*, 2014).

Evaluation of antioxidant properties showed that the pattern of increasing antioxidant power in leaves and seeds was positively correlated with total flavonoid, rutin, and quercetin. Under osmotic stress such as salt and drought, all physiological behaviour probably be affected (Elnaggar *et al.*, 2020). The flawed protecting system against high amounts of ROS due to oxidative stress may damage macromolecules such as DNA, proteins, and membrane lipids (Plaza *et al.*, 2009). As part of the antioxidant defence, lipid peroxidation inhibitors, and ROS scavenging system, the nonenzymatic compounds like phenols and flavonoids are electron/hydrogen donors and therefore could extenuate the effect of oxidative stress (Oh *et al.*, 2009). As part of the phenylpropanoid system, flavonoids serve in the scavenging of free radicals via reducing them with hydroxyl groups existing in their structure (Pourcel *et al.*, 2007; Han *et al.*, 2012). The degree of oxidation and saturation in the heterocyclic ring could determine different kinds of flavonoids such as flavones (flavone, luteolin), flavonols (quercetin, kaempferol, myricetin), flavanones (flavanone, naringenin), and others (Pourcel *et al.*, 2007). In an interesting study, Iqbal *et al.* (2021) observed the enhancement of antioxidant and DNA damage inhibition potentials of *N. sativa* callus treated under a high dose of sodium azide that could be related to the elicitation of other secondary metabolites or the formation of thymoquinone analog(s) with more potent antioxidant activity. Regarding this new finding, a more thorough molecular study and different enzymatic/non-enzymatic antioxidant agents are needed under different doses of NaCl. The evidence of the overexpression of some genes related to flavonoid metabolism of *Arabidopsis thaliana* under drought conditions supported the high antioxidant activity of flavonoids (Nakabayashi *et al.*, 2014). Based on Toma *et al.* (2015), there are nineteen phenolic and flavonoid compounds, including caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, sinapic acid, cichoric acid, hyperoside, isoquercitrin, rutin, myricetin, fisetin, quercitrin, quercetin, patuletin, luteolin, kaempferol, and apigenin in the ethanolic extracts of *N. sativa*.

Our finding indicated that flavonols components were affected more at salinity conditions rather than drought stress. In the literature, Golkar *et al.* (2020) have also recorded a similar increase of antioxidant activity in *N. sativa* callus treated by intensive salinity related to the enhancement of total anthocyanin's concentrations and the total flavonols content.

Some studies indicated no difference between rutin and quercetin under drought stress Gray *et al.* (2003): *Hypericum perforatum*). It was reported that there is a positive correlation between antioxidant activity and rutin and quercetin content (Azarafshan *et al.*, 2020). Comparing the amount of quercetin and kaempferol in treated leaves and seeds showed that their amount in the leaves is more than the seeds. In contrast, the amount of kaempferol in seeds is more significant than the leaves. It seems that kaempferol and quercetin indicated more increase in response to salinity treatments. In the seed, the more enhancement of kaempferol than quercetin indicated that biosynthesis of flavonoids shifted toward kaempferol. The greater antioxidant power of kaempferol may explain this to quercetin (Amić *et al.*, 2003). A more significant amount of kaempferol than quercetin was reported in *Arabidopsis thaliana* shoots and roots under drought stress (Shojaie *et al.*, 2016). Moreover, the diversity in flavonoid components such as quercetin in leaves of *Ligustrum vulgare* under drought stress was recorded (Tattini *et al.*, 2004).

As a flavonol component, rutin plays protecting role against lipid peroxidation of membrane via interaction with the polar head of phospholipids (Erlejmán *et al.*, 2004). The presence of catechol group on the B ring of flavonoids leads to produce radicals with more stability than those are flavones like rutin produced. This makes the rutin weaker than quercetin and kaempferol (Amić *et al.*, 2003). However, the results indicated that the black cumin probably preferred the accumulation of rutin over kaempferol and quercetin in leaves and seeds. Thus, it seems that rutin is not the main reason for high antioxidant activity, at least in seeds treated by NaCl 60 mM.

The accompanying increase in phenol/flavonoid contents in seeds under 2DI and NaCl 30mM treatments supports this concept that defence systems against abiotic stress in plants effectively generate secondary metabolites. While the seeds were relatively sensitive at high salinity treatment (NaCl 60 mM), a

significant decrease was observed in antioxidant activity, rutin, kaempferol, and myricetin values. Regarding the importance of seeds of *N. sativa* in medicinal and foodstuff, this could be beneficial in related industries.

Conclusions

The present study aimed to examine that salinity and drought stress has a remarkable influence on the phenol, flavonoid, and antioxidant activity of *N. sativa*. The present study shows that all physiological behavior of *N. sativa* was affected under salt and drought stress. The highest amount of total phenol, flavonoids, and carotenoid content of leaves were increased under salinity and drought stresses. The evaluation of antioxidant properties provided evidence that there is a symmetrical pattern between increasing the antioxidant capacity with the total flavonoid, rutin, and quercetin. Results indicated a higher sensitivity of *N. sativa* to drought stress than salinity stress. Furthermore, our survey indicated that moderate salinity and drought could enhance secondary metabolites in *N. sativa* seeds as important medicinal plant organs. This would be interested in pharmacological industries in progressing novel chemotypes regarding the intensity of salinity and drought stress.

Authors' Contributions

MP and AM conceived and designed the research. Also, they analyzed and interpreted the data. SA-G performed the experiments. MP and SA-G wrote the manuscript. HA contributed in preparing reagents, materials, analysis tools or data.

All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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