Humic acid affects some growth parameters, chlorophyll, flavonoids, antioxidant enzymes, and essential oil of *Satureja khuzestanica* Jamzad under salinity stress

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Abstract

Salinity stress is a limiting factor for plant growth and production. To reduce the salinity effects, humic acid was applied in soil, and its mitigation effect was studied on *Satureja khuzistanica* Jamzad. The research was conducted as a factorial experiment based on a completely randomized block design with four replications at Lorestan University. The factors included salinity stress (0, 25, 50, 75, and 100 mM NaCl) and humic acid (0, 10, 20, 30, and 40 mg/kg soil). Results showed that salinity stress decreased plant height, dry weight, leaf number, root length, chlorophyll, and carotenoids. However, it significantly increased flavonoid contents and SOD, CAT, and GR activities. Essential oil (EO) quantity and quality were affected by salinity. The EO percentage and yield decreased by salinity. Forty-three constituents were identified, among which carvacrol, γ-terpinene, α-terpinene, myrcene, p-cymene, α-thujene, citronellol, and α-pinene were the major compounds according to their contents and formed more than 94% of the EO. The percentage of some of the other constituents decreased to zero under specific salinity and humic acid treatments. The application of humic acid could mitigate salinity effects on growth and EO production. However, there was an interaction between salinity and humic acid on EO yield and quality. It seems that the application of humic acid is useful for improvement EO yield of *S. khuzestani*, but the amount of its application depends on the EO consumers' favorite.

Keywords: antioxidant, medicinal plants, organic essential oil, environmental stress, secondary metabolites

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Introduction

About a third of the drugs have a natural and herbal origin, and the pharmaceutical industry is trying to minimize the production of chemical drugs and rely more on herbal medicines. More than 60 percent of the world's herbal medicines are made from medicinal plants found in natural habitats. This rate of harvesting of medicinal plants without their planting will expose them to extinction. The most suitable method to help preserve medicinal plants and survive species in natural habitat is the intensive production of these species by farmers (Najafi et al., 2010). Besides, the organic production of medicinal plants should be considered as the use of

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chemical fertilizers has side effects on the quality of medicinal products.

Iran has a rich flora due to its high climatic diversity. There are more than 8,000 plant species in the Iranian flora, of which about 2,000 are endemic. In this flora, there are important medicinal plants, including *Satureja* species belonging to the genus Mint. This genus has 14 species of annual and perennial herbaceous plants in Iran, nine of which are endemic. One of them is the *S. khuzestanica* Jamzad (Mozafarian, 1996; Rechinger, 1982).

Different studies reported different EO constituents for *S. khuzestanica*. For example, Saei-Dehkordi et al. (2012) reported 28 components, among which monoterpenes were the main and carvacrol (53.86%) and thymol (19.84%) were the most abundant. The EO had satisfactory antimicrobial activity against most of the studied microorganisms.

Some other studies have reported antimicrobial properties for savory oil. In vitro anti-fungal effects have been reported for savory essential oil (Essawi and Srour, 2000; Sokovic et al., 2002; Yazdanpanah et al., 2010). Yazdanpanah et al., 2010 reported that savory essential oil at 400 ppm or more inhibited growth of *Alternaria citri*; however, low concentrations (below 400 ppm) only slowed down the growth and could not hinder it. However, Rahimian and Eisvand (2016) compared antifungal effects of *S. khuzestanica* oil at 10 and 20 ppm with Vitavax (a chemical fungicide) 2 g per kg to control *Fusarium oxysprum* infection in wheat seed in a pot experiment. They reported that the antifungal effect of savory was not as effective as that of Vitavax.

The amount and quality of secondary metabolites are affected by environmental stresses. Drought, salinity, cold, and heat are the most significant abiotic stresses, leading to a global decline in crop production (Nakabayashi and Saito, 2015). Essential oil yield is reduced under stressful conditions and needs to be improved with management operations such as optimal plant nutrition, particularly appropriate, i.e. organic fertilizers. Due to global climate change, one of the expected dangers is the increase in the salinity of agricultural lands. By 2050, more than 50 percent of the world's land is expected to be affected by salinity stress (Nimir Eltyb et al., 2015).

Humic fertilizers play central roles in farming systems through sustainable nutrients provided to plants and improving soil physicochemical conditions. In addition, they supply energy for soil microorganisms and thus have a decisive influence on chemical, physical, and biological properties of the soil, thereby exerting direct impacts on plant growth (Quaggiotti et al., 2004). Humic acid is used as a suitable fertilizer in organic farming. The beneficial effects of this fertilizer under saline conditions include positive effects on the soil physicochemical properties, stimulating bacterial growth, increased access to nutrients, providing organic and amino acids (Khattak et al., 2013), and alkaline soil pH adjustment (Karakut et al., 2009). Due to its strong chelating properties, humic acid retains sodium ions and prevents them from being absorbed by the plant. The ability of humic substances to stimulate root growth and chelate ions has led to one of the most commonly reported benefits of humic acids, viz. increased nutrient uptake by plants (Nardi et al., 2009).

Salinity (NaCl) stress severely reduces the growth parameters of savory (leaf area and dry weight) and pigment content (chlorophyll and carotenoids) while increasing proline and soluble sugar (Najafi et al., 2010). Salinity was also reported to decrease photosynthetic pigments (Amiri and Moazzeni, 2016) and photosynthesis rate of *S. khuzestanica* (Zaremanesh et al., 2019). On the other hand, application of humic acid was reported to mitigate the effect of salinity on plant height and shoot and root dry weights (Khosropour et al., 2015).

In addition to the therapeutic aspect, medicinal plants are also considered one of the potentials for economic development. If they are studied well and are allowed to be cultivated in marginal areas (unsuitable lands exposed to salinity and drought stresses), they will generate significant income. Increasing the production of *S. khuzestanica* organically and improving the quantity and quality of its oil in control and salinity stress conditions using humic acid were

Table 1
Soil characteristics before applying the treatments (humic acid and salinity)

Soil texture	pH	Electrical conductivity (ds m^{-1})	Cation exchange capacity	Bulk density (g/cm^3)	Organic matter (%)	Organic carbon (%)	N (%)	P (mg/kg soil)	K (mg/kg soil)	Mn (mg/kg soil)	Cu (mg/kg soil)	Zn (mg/kg soil)	Fe (mg/kg soil)	Mo (mg/kg soil)
Sandy clay loam	7.02	0.38	11.30	1.28	1.78	1.18	0.11	44.30	280.21	3.01	2.95	0.91	9.08	0.75

the goals of this study. The aim was to study the probable alleviating impact of humic acid on *S. khuzestanica* under salinity conditions as well as its effects on essential oil profiles, antioxidant enzymes, flavonoids, and some morpho-physiological traits of this plant.

Materials and Methods

Seed and other experiment materials

Satureja (*S. khuzistanica* Jamzad) seeds were obtained from the Gene Bank of the Forests and Rangelands Research Institute, and humic acid fertilizer was procured from Sarvestan Pak Iranian Company (Spi). The soil consisted of a ratio of field soil (2): sand (1) and sheep manure (0.5). The soil physicochemical properties are given in Table 1. The pots were polyethylene (21 cm in diameter and 20 cm high) that contained 4 kg soil.

The study was performed as a pot experiment in 2017 in the Greenhouse of Lorestan University, Iran. Its statistical model was a factorial based on a randomized complete block design (RCBD) with four replications. Twenty seeds were planted in each pot. Due to the small size of the seeds, they were planted at a depth of about 1-2 cm. The pots were irrigated after planting, and covered with plastic to ensure rapid and uniform germination. Two pots were considered for each repetition, one for essential oil extraction and the other for morphological and physiological traits. At the 4-leaf stage, thinning was done so that there were 10 plants left in each pot. The factors included in the current research were humic acid (0, 10, 20, 30, and 40 mg/kg soil) incorporated into soil before planting; and salinity stress (0, 25, 50, 75, and 100 mM NaCl) which was applied after seedling establishment at the fourth leaf stage. Irrigation was done twice a week. Treatment abbreviations are shown in Table 2.

Table 2
Treatment abbreviations

Salinity (mM NaCl)	Humic acid (mg/kg soil)
S1=0	H1=0
S2=25	H2=10
S3=50	H3=20
S4=75	H4=30
S5=100	H5=40

Measured traits

Five plants were selected randomly at flowering stage and harvested to measure some morphological traits including plant height, root length, number of secondary branch, number of leaf, leaf area, and shoot dry weight (leaf and flowering branch, which are considered oil bearing). Drying was done immediately at room temperature in shade (Hadian et al., 2010).

Essential oil extraction and analysis

At full flowering stage, a 10-gram sample of shoot was dried and powdered from each pot. Then, essential oils were extracted using a Clevenger apparatus (EMO500/C model) according to the method recommended in British pharmacopoeia,

1993. The collected essential oil was weighed and also calculated based on milliliters. Then oil containers were sealed with foil and kept at 4 ℃ in the refrigerator until analysis. The EO percentage and yield were calculated based on the following formulas:

EO (%) = (EO weight/Plant sample weight) × 100

EO yield (ml/plant) = Plant weight × EO volume (ml) extracted from 10 g/10 g

To carry out GC-MS analysis, a Shimadzu model GC-17A (Kyoto, Japan) gas chromatograph coupled to a Shimadzu Quadruple-MS model QP5050 mass spectrometer was used. Compounds were separated on a 30 m × 0.22 mm ID fused-silica capillary column coated with 0.25 µm film of BP-5 (Shimadzu) and a split injector with a 1 mm internal diameter glass liner. Ultra-pure helium was used as carrier gas with ionization voltage of 70 eV. The carrier gas flow rate was 1 ml/min; the oven temperature program was 40–100 ℃ at the rate of 20 ℃/min. It was then raised to 220 ℃ with a rate of 3 ℃ min^{-1} and finally raised to 280 ℃ with a rate of 30 ℃ min^{-1} and kept at this point for 5 min.; split ratio was 1:100. Injector and interface temperatures were 280 ℃ and 260 ℃, respectively. Mass ranged from 35 to 450 amu. The essential oil constituents were identified using calculation of their retention indices under temperature-programmed conditions for n-alkanes (C8–C20) and the oil on a DB-5 column under the same chromatographic conditions. Identification of compounds was performed by comparison of their mass spectra with those of the internal reference mass spectra library (NIST08 and Wiley 9.0).

Chlorophyll and carotenoids

Fresh plant tissue (0.1 g) was homogenized in a porcelain mortar with liquid nitrogen. After adding 10 ml of 80% acetone, the homogenized mixture was centrifuged for 10 min at 6000 rpm. Then the supernatant was separated and the absorption was read by the spectrophotometer at 663 nm for chlorophyll a, 645 nm for chlorophyll b, and 470 nm for carotenoids. Using the following formulas, chlorophyll a, b, and carotenoids were estimated (Arnon, 1967).

Chlorophyll a (mg/g FW) = $(19.3 \times A_{663} - 0.86 \times A_{645})$ V/100W

Chlorophyll b (mg/g FW) = $(19.3 \times A_{645} - 3.6 \times A_{663})$ V/100W

Carotenoids (mg/g FW) = $100(A_{470})$ - 3.27(mg Chl. a) - 104(mg Chl. b)/227

where *A* = absorbance at specific wavelengths, *V* = final volume of chlorophyll extract, and *W* = fresh weigh of tissue extracted

Total flavonoids

The flavonoid content was measured by aluminum chloride coloring method. Accordingly, 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of potassium acetate 1M, and 2.8 ml of distilled water were added to 0.5 ml methanolic leaf extract which was then stored at room temperature for 30 minutes. Finally, its absorption was read at 415 nm by the spectrophotometer (Photonix Ar 2015 model). Different concentrations of quercetin 0-400 µg/ml methanol were used to draw the standard curve (Chang et al., 2002).

Total protein

Leaf tissue (0.1 g) was powdered by liquid nitrogen then homogenized with 1 ml of phosphate buffer (100 mM, pH 7.8), containing EDTA (1 mM), DTT (4 mM), and 10% glycerol, in a mortar which was placed on ice. The resulting mixture was centrifuged at 13000 rpm for 20 min at 4 ℃. The supernatant was used to measure the total soluble protein by the Bradford method (Bradford, 1976).

Antioxidant enzymes assay

At flowering stage, half a gram of leaf sample was well powdered with liquid nitrogen in a mortar and completely homogenized with phosphate buffer containing 1 mM EDTA, 1 mM PMSF, and 1% PVP-40. It was then centrifuged at 15000 rpm for 10 min at 4 ℃ and the supernatant was used to measure the activity of antioxidant enzymes (Sudhakar, 2016).

Table 3
ANOVA (mean square) for the effect of salinity and humic acid on some morphological traits of *S. khuzestanica* Jamzad.

SOV	df	Plant height	Root length	Number of secondary branches	Leaf no. per plant	Shoot dry weight (g/plant)
Replication	3	3.11 ^{ns}	5.08*	15.79**	21.40 ^{ns}	0.015**
Salinity (S)	4	279.00**	16.02**	79.02**	3939.00**	0.19**
Humic acid (H)	4	83.10**	30.25**	733.66**	7147.00**	0.29**
S × H	16	0.64^{ns}	0.035^{ns}	0.23^{ns}	0.002^{ns}	0.00000008^{ns}
Error	73	1.82	1.26	2.95	11.18	0.0026
CV (%)		5.27	7.10	7.62	2.80	5.75

ns,*, and ** represent non-significant, significant at p≤0.05, and p≤0.01, respectively.

CAT activity

Catalase activity was assayed according to Dhindsa et al. (1981) method. Accordingly, 100 µl of the enzymatic extract was mixed with 1900 µl reaction buffer containing 50 mM of potassium phosphate buffer (pH=7) and 15 mM H_2O_2. The absorption was read by a spectrophotometer for one minute at 240 nm.

POD activity

POD activity was assayed according to Chance and Maehly (1955). In this method, 33 µl of enzyme extract was mixed with 1 ml of reaction buffer containing 13 mM guaiacol, 5 mM H_2O_2 and 50 mM potassium phosphate buffer (pH=7), and its absorption was read by a spectrophotometer at 470 nm at 10 second intervals for 1 min.

SOD

SOD activity was assayed according to Beauchamp and Fridovich (1971). In this method one ml of mixture containing 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 14.53 mM methionine, and 82.5 µM NBT was added into a glass cuvette, followed by adding 20 µl of enzyme extract. Reaction was started by adding 2.2 µl of riboflavin solution, and the absorbance of solution was measured at 560 nm. SOD activity was expressed as U (unit) mg^{-1} protein. One unit of enzyme activity is amount of enzyme required for 50% inhibition of NBT reduction at 560 nm.

GR

The GR activity was assayed according to Rios-Gonzalez et al. (2002). One ml of reaction mixture was prepared including 0.1 M Tris buffer (pH=7.8), 2 mM EDTA, 50 µM NADPH, 0.5 mM GSSG (oxidized glutathione), and 20 µl of enzyme extract. The assay was started by adding NADPH at 25 ℃ and absorption was read by spectrophotometer at 340 nm for 1 minute.

Statistical Analysis

Checking data for out-layers and normality test were carried out by Minitab software. Data analysis was done using SAS V. 9.4 and means were compared using Duncan's multiple range test. Excel software was used for drawing the graphs.

Results

Plant height and root length

Salinity decreased plant height. On the other hand, humic acid increased this trait (Tables 3 and 4). Root length also was affected by salinity (Table 3) and decreased (Table 4). Although there was a decreasing trend in root length with an increase in salinity level, the effect was not significant at 25, 50, and 75 mM NaCl (Table 4).

Number of secondary branches and leaves

Salinity decreased number of secondary branches, and this decrease was significant from 50 mM NaCl onward. Humic acid increased secondary branches significantly (Tables 3 and 4). Leaf number was profoundly affected by both salinity and humic acid (Table 3). It was decreased by salinity while humic acid increased number of leaves significantly (Table 4).

Shoot dry weight

The highest shoot dry weight was observed in S1H5 treatment with 2.04 g/plant. Although humic acid could mitigate salinity effects on dry

Table 4
Mean comparison for the effect of salinity and humic acid on some morphological traits of *S. khuzestanica* Jamzad

Treatments	Plant height (cm)	Root length (cm)	Secondary branch no.	Leaf no. per plant	Shoot dry weight (g/plant)
Salinity (mM NaCl)					
0	29.9 a*	17.1 a	24.6 a	138.5 a	1.89 a
25	28.1 b	16.3 b	24.0 a	124.3 b	1.89 a
50	26.2 c	15.6 bc	22.8 b	119.7 c	1.83 b
75	22.7 d	15.2 dc	21.8 b	113.2 d	1.80 b
100	20.9 e	14.8 d	19.6 c	100.3 e	1.67 c
Humic acid (mg/kg soil)					
0	23.1 e	14.21 d	20.3 d	96.4 e	1.67 e
10	24.2 d	15.01 c	21.2 d	107.8 d	1.75 d
20	25.4 c	15.99 b	22.4 c	116.1 c	1.82 c
40	26.8 b	16.69 ab	23.6 b	132.2 b	1.87 b
60	28.3 a	17.24 a	25.1 a	143.7 a	1.97 a

* in each horizontal section: means in each column with at least one common letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Table 5
ANOVA (mean square) of the effect of salinity and humic acid on some physiological traits of *S. khuzestanica* Jamzad

SOV	df	Chl a	Chl b	Carotenoid	Flavonoid content	SOD activity	CAT activity	POD activity	GR activity
Rep.	3	0.000003	0.000002	0.000001	0.08	0.000003	0.014	0.000001	0.000005
Salinity (S)	4	0.50**	0.12**	0.24**	87.10**	0.006**	0.27**	0.0002**	0.00029**
Humic acid (H)	4	0.033**	0.012**	0.016**	15.95**	0.33**	1.65**	0.003**	0.0035**
S × H	16	0.0001**	0.00007**	0.000056**	0.44**	0.0000007^{ns}	0.0007^{ns}	0.000008**	0.000009**
Error	73	0.000001	0.000002	0.000004	0.02	0.000003	0.011	0.000001	0.000003
CV (%)		0.16	0.49	0.34	1.07	0.20	6.03	0.84	6.00

Ns, *, and ** represent non-significant, significant $p \leq 0.05$, and significant $p \leq 0.01$, respectively.

weights, S3H5 and S2H5 treatments were not significantly different from S1H5. Salinity decreased dry weight and the minimum dry weight (1.5 g/p) resulted from the S5H1 treatment (Table 4).

Chl a and Chl b

Results showed that the interaction of the effects of salinity and humic acid on chlorophylls was significant (Table 5). The S1H5 treatment had the highest levels of chlorophyll a and b, and the lowest level was observed in S5H1 treatment (Table 6). In addition to increasing the amount of chlorophyll in control conditions, humic acid also prevented the harmful effects of salinity on chlorophyll reduction (Table 6).

Carotenoids

Salinity reduced the carotenoids contents while the use of humic acid mitigated salinity and increased these pigments. The highest carotenoids content was observed in S1H5 treatment with an average of 0.7 mg/g dry weight. The lowest one was recorded in S5H1 treatment, with an average of 0.39 mg/g dry weight (Table 6).

Flavonoids

The interaction between salinity and humic acid was significant on the flavonoids content (Table 5). With increasing salinity and humic acid, flavonoid levels increased, but this trend was not the same at every level of salinity. The highest levels of flavonoids were observed in S5H5 treatment, but control (S1H1 treatment) had the lowest value (Table 6).

SOD

The effect of salinity and humic acid were significant on SOD activity (Table 5). SOD activity increased under both salinity and humic acid treatments (Fig. I).

Table 6
Mean comparison for the interaction of salinity and humic acid on some physiological traits of *S. khuzestanica* Jamzad

Salinity (mM NaCl)	Humic acid (mg/kg soil)	Chl a (mg/g FW)	Chl b (mg/g FW)	Carotenoids (mg/g FW)	Flavonoids (mg/g FW)
0	0	0.829 g	0.385 g	0.656 g	8.27 s
	10	0.840 f	0.400 e	0.667 f	9.45 q
	20	0.872 d	0.420 c	0.686 d	11.29 n
	40	0.913 b	0.448 b	0.715 b	13.06 j
	60	0.938 a	0.461 a	0.734 a	14.37 g
25	0	0.777 k	0.366 k	0.625 i	8.62 r
	10	0.791 i	0.378 i	0.633 h	10.04 p
	20	0.822 h	0.391 f	0.654 g	11.70 m
	40	0.858 e	0.408 d	0.677 e	13.66 i
	60	0.876 c	0.421 c	0.694 c	15.33 f
50	0	0.684 o	0.327 n	0.563 m	9.39 q
	10	0.698 n	0.340 m	0.581 l	10.81 o
	20	0.731 m	0.351 l	0.602 k	12.35 k
	40	0.754 l	0.369 j	0.619 j	14.05 h
	60	0.782 j	0.383 h	0.632 h	16.34 d
75	0	0.563 t	0.265 r	0.474 r	11.48 n
	10	0.587 s	0.277 q	0.492 q	13.06 j
	20	0.611 r	0.290 p	0.508 p	15.35 f
	40	0.639 q	0.313 o	0.522 o	17.59 c
	60	0.669 p	0.329 n	0.540 n	18.42 b
100	0	0.446 y	0.197 w	0.388 w	12.08 l
	10	0.459 x	0.209 v	0.401 v	13.86 hi
	20	0.481 w	0.228 u	0.423 u	16.09 e
	40	0.506 v	0.241 t	0.442 t	18.29 b
	60	0.531 u	0.259 s	0.462 s	19.74 a

*Means in each column with at least one common letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

CAT

Effects of salinity and humic acid were significant on the CAT activity (Table 5). It increased by both salinity stress and humic acid treatments (Fig. II).

POD

Interaction between salinity and humic acid had significant effects on POD activity (Table 5). The highest peroxidase activity was observed in S4H5 treatment and the lowest one was recorded in control, i.e. S1H1 treatment (Table 7).

GR

Interaction between humic acid and salinity was significant on GR activity (Table 5). Treatment S5H5 induced maximum GR activity (Table 7).

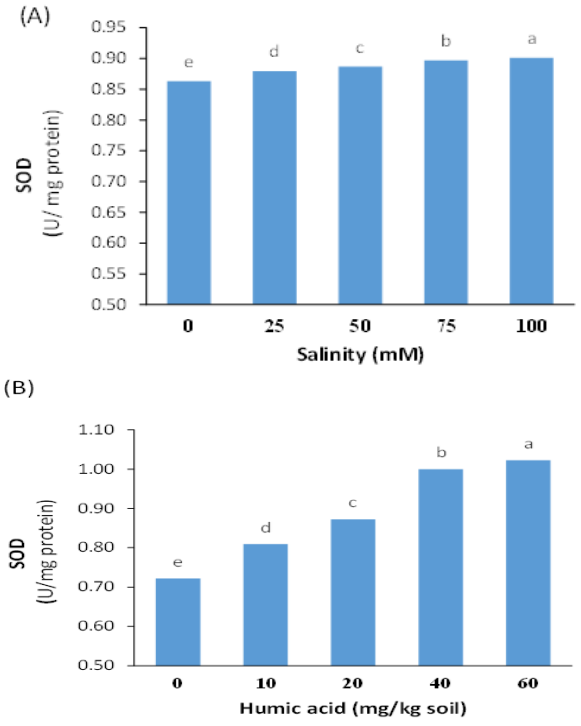

Fig. I. Effect of salinity (A) and humic acid (B) on SOD activity of *S. khuzestanica* Jamzad.

Table 7
Means comparison for the interaction of effects of salinity and humic acid on POD and GR in *S. khuzestanica* Jamzad

Salinity (mM NaCl)	Humic acid (mg/kg soil)	POD (U/mg protein)	GR (U/mg protein)
0	0	0.1213 n	0.0100 k
	10	0.1280 k	0.0200 i
	20	0.1340 hi	0.0230 h
	40	0.1503 e	0.0399 d
	60	0.1543 c	0.0455 b
25	0	0.1235 m	0.0111 k
	10	0.1290 k	0.0191 i
	20	0.1330 ij	0.0232 h
	40	0.1470 f	0.0345 e
	60	0.1500 e	0.0415 cd
50	0	0.1258 l	0.0146 j
	10	0.1315 j	0.0199 i
	20	0.1350 h	0.0254 gh
	40	0.1493 e	0.0393 d
	60	0.1525 d	0.0440 bc
75	0	0.1288 k	0.0187 i
	10	0.1338 hi	0.0262 gf
	20	0.1385 g	0.0268 gf
	40	0.1548 bc	0.0452 b
	60	0.1608 a	0.0505 a
100	0	0.1290 k	0.0203 i
	10	0.1343 hi	0.0263 gf
	20	0.1395 g	0.0287 f
	40	0.1560 b	0.0459 b
	60	0.1613 a	0.0527 a

*Means in each column with at least one common letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Essential oil percentage and yield

The effect of salinity and humic acid on the percentage of EO was significant (Table 8). EO percentage decreased by increasing salinity. The use of humic acid higher than 10 mg/kg soil increased the EO percentage (Table 9). The yield of EO was affected by the interaction of salinity and humic acid (Table 8). Using humic acid could mitigate the adverse effects of salinity on the EO yield. Therefore, maximum EO yield was produced under control condition and 60 mg/kg soil of humic acid, i.e. S1H5 treatment (Fig. V).

Oil constituents

The results of GC and GC-MS analysis of EO identified 43 compounds in *S. khuzestanica*, some of the most important of which, including 17 compounds, were statistically analyzed in Table 8. Eight compounds with at least one percent and a total of more than 94% of EO included carvacrol, γ-terpinene, α-terpinene, myrcene, p-cymene, α-

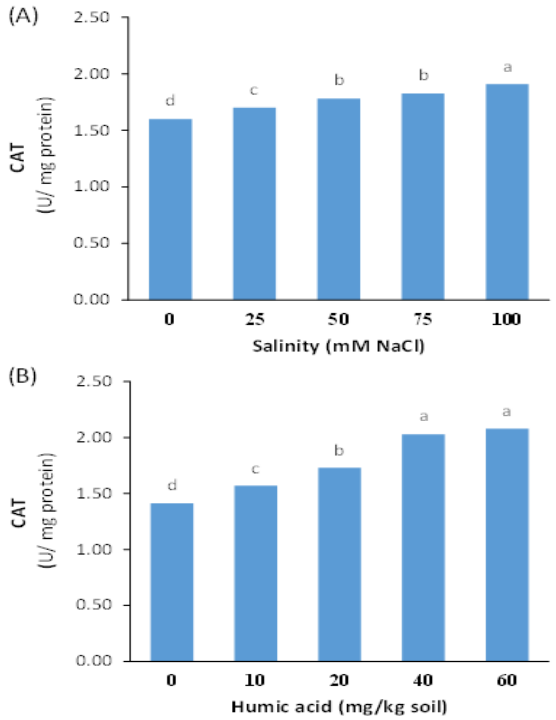

Fig. II. Effect of salinity (A) and humic acid (B) on CAT activity of *S. khuzestanica* Jamzad.

Table 8
ANOVA (mean square) of the effect of salinity and humic acid on essential oil percent, yield, and constituents of *S. khuzestanica* Jamzad

SOV	df	Essential oil percent	Essential oil yield	Carvacrol	gamma terpinene	Alpha-Terpinene	Myrcene	p-cymene	alpha thujene	Citronellol	alpha pinene
Replication	3	0.0031^{ns}	0.000003**	1.42*	3.09**	0.09**	0.17**	0.02**	0.022^{ns}	0.011**	0.031^{ns}
Salinity (S)	4	0.46**	0.0003**	1167.80**	475.10**	22.5**	12.74**	4.76**	2.54**	2.09**	0.73**
Humic acid (H)	4	0.46**	0.00032**	317.00**	121.20**	16.11**	15.44**	4.77**	4.26**	0.33**	1.22**
S×H	16	0.0086^{ns}	0.00000005**	948.60**	453.60**	24.9**	12**	4.26**	3.42**	1.01**	1.21**
Error	72	0.0012	0.0000001	0.50	0.50	0.01	0.007	0.002	0.013	0.0016	0.012
CV (%)		3.5	1.57	1.59	2.11	2.24	2.31	2.03	5.93	3.36	9.34

SOV	df	beta bisabolene	Limonene	Caryophyllene	beta phellandrene	Thymol	Camphene	beta trans ocimene	Borneol	alpha phellandrene	alpha pinene
Replication	3	0.02**	0.0010^{ns}	0.009^{ns}	0.02*	0.01^{ns}	0.003**	0.003**	0.002**	0.007*	0.031^{ns}
Salinity (S)	4	0.20**	0.13**	0.55**	0.08**	1.0**	0.02**	0.09**	0.019**	0.50**	0.73**
Humic acid (H)	4	0.98**	0.30**	0.35**	0.044**	1.92**	0.017**	0.034**	0.008**	0.43**	1.22**
S×H	16	0.86**	0.24**	1.55**	0.099**	0.80**	0.03**	0.10**	0.01**	0.40**	1.21**
Error	72	0.0016	0.007	0.0037	0.005	0.01	0.0005	0.0004	0.0003	0.001	0.012
CV (%)		6.30	14.74	11.37	17.53	33.84	15.31	13.46	15.95	6.66	9.34

ns, *, and ** represent non-significant, significant at p≤0.05, and significant at p≤0.01, respectively.

thujene, citronellol, and α-pinene. Carvacrol and γ-terpinene were the two main compounds that made up more than 70% of the essential oil (Fig. III).

These two constituents showed a different response to the applied treatments, so that a significant interaction between salinity and humic acid was found on carvacrol and γ-terpinene contents (Table 8). The lowest and highest levels of carvacrol were observed in S2H4 and S1H5 treatments with averages of 28.5% and 81.8%, respectively. However, the highest γ-terpinene was seen in S1H5 treatment with an average of 52.3%; this reached its lowest value, i.e. 10.9%, in S4H2 treatment (Table 9). Alpha-terpinene was

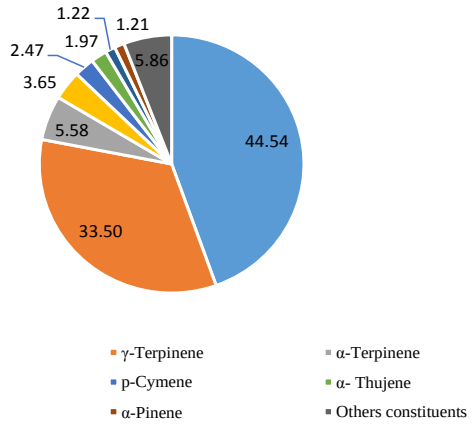

Fig. III. The major compounds of essential oil in *S. khuzestanica* Jamzad

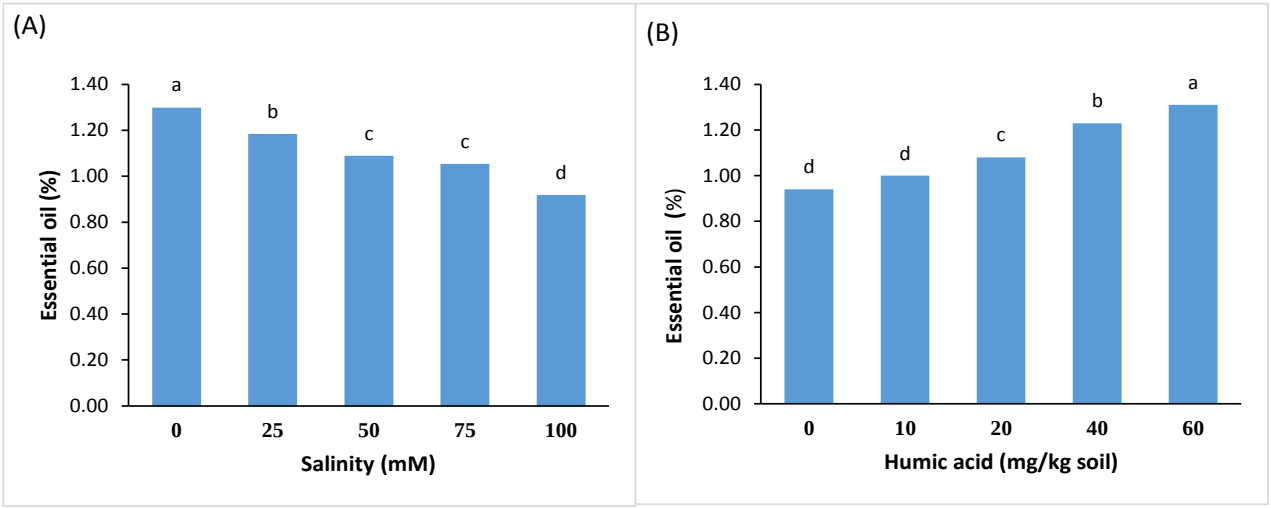

Fig. IV. Effects of salinity and humic acid on essential oil percent in *S. khuzestanica* Jamzad

Table 9
Mean comparison for the interaction of salinity and humic acid on 17 constituents of essential oil

Salinity (mM NaCl)	Humic acid (mg/kg soil)	Carvacrol	γ-Terpinene	α-Terpinene	Myrcene	p-Cymene	α- Thujene	Citronellol	α-Pinene	α-Phellandrene
	0	36.9 kl*	40.9 c	6.61 g	4.60 d	2.69 g	2.01 h	0.93 j	1.17 i	0.66 i
	10	39.8 h	41.4 c	5.20 j	3.36 fg	1.93 k	1.52 i	2.03 b	0.87 j	0.49 j
0	20	35.7 m	37.9 d	6.31 h	4.48 d	3.47 c	2.66 ef	1.87 c	1.34 gh	0.87 f
	40	37.6 jk	33.1 h	7.53 cd	5.84 a	3.98 a	3.02 b	0.49 m	1.92 ab	1.00 c
	60	28.5 o	52.3 a	5.58 i	3.42 fg	2.12 j	1.48 i	2.11 a	0.80 jk	0.52 j
	0	38.4 ij	32.0 i	7.69 c	5.84 a	3.61 b	2.76 c-e	2.02 b	1.62 de	0.91 ef
	10	39.2 hi	42.6 b	5.72 i	3.37 fg	2.15 j	1.58 i	0.33 n	0.72 jk	0.53 j
25	20	52.3 d	38.1 d	1.27 n	0.60 k	1.20 n	0.46 kl	1.39 e	0.82 j	0.62 i
	40	81.8 a	11.1 n	0.34 p	0.48 l	0.43 p	0.33 l	1.60 d	0.22 m	0.12 l
	60	36.4 ml	35.1 g	8.03 b	5.18 c	2.87 f	2.64 ef	1.05 hi	1.43 gf	0.80 g
	0	33.1 n	34.6 g	8.37 a	5.95 a	3.62 b	3.25 a	1.59 d	1.87 bc	0.94 de
	10	41.8 g	34.9 g	5.11 j	3.47 f	2.51 h	1.51 i	1.55 d	1.44 gf	0.51 j
50	20	38.1 j	37.6 de	7.29 e	3.35 g	2.76 g	2.06 h	1.56 d	1.31 g-i	0.80 g
	40	43.5 f	31.6 ij	6.59 g	4.51 d	3.16 e	2.84 cd	1.28 f	1.25 hi	0.74 h
	60	36.1 ml	30.9 j	7.22 e	5.53 b	3.14 e	2.54 gf	1.04 hi	1.46 e-g	0.98 cd
	0	33.0 n	36.3 f	8.17 b	5.93 a	3.67 b	3.05 b	1.09 h	1.96 ab	1.02 c
	10	81.2 a	10.9 n	0.93 o	0.46 l	0.41 p	0.30 l	1.58 d	0.24 ml	0.11 l
75	20	37.7 jk	40.6 c	6.96 f	3.06 h	2.54 h	2.13 h	1.38 e	1.21 hi	0.79 gh
	40	27.8 o	51.3 a	6.21 h	4.22 e	2.74 g	2.17 h	1.22 g	1.31 g-i	0.63 i
	60	43.4 f	25.9 k	6.24 h	4.24 e	3.32 d	2.47 g	1.06 hi	1.74 cd	1.18 a
	0	62.9 c	24.9 l	2.76 l	1.77 j	1.31 m	1.05 j	1.01 i	0.65 k	0.32 k
	10	49.0 e	36.6 ef	4.06 k	2.32 i	1.50 l	1.20 j	0.32 n	0.78 jk	0.36 k
100	20	42.3 g	31.9 i	6.26 h	3.47 f	2.36 i	2.74 de	0.69 k	1.57 ef	0.08 l
	40	43.8 f	24.7 l	7.52 d	5.25 c	3.6 b	2.92 bc	0.70 k	2.08 a	1.09 b
	60	71.3 b	20.4 m	1.59 m	0.62 k	0.74 o	0.50 k	0.61 l	0.39 l	0.14 l

Table 9. Continued

Salinity (mM NaCl)	Humic acid (mg/kg soil)	β-Bisabolene	Limonene	Caryophyllene	β-Phellandrene	Thymol	Camphene	β-trans-Ocimene	Borneol
0	0	0.49 i	0.52 gh	0.76 e	0.38 e-i	0.00 h	0.13 ij	0.15 i	0.07 m-l
	10	0.56 h	0.48 hi	0.66 fg	0.32 j-i	0.00 h	0.08 k	0.15 i	0.05 op
	20	0.71 f	0.69 d-f	0.72 ef	0.43 d-g	0.19 f-h	0.25 b	0.25 g	0.05 orn
	40	0.78 e	0.81 a-d	0.76 e	0.59 ab	0.15 gh	0.27 ab	0.36 bc	0.12 ih
	60	0.31 k	0.47 hi	0.00 j	0.35 g-i	0.46 de	0.12 jk	0.32 de	0.00 q
25	0	0.78 e	0.78 b-e	0.87 d	0.54 a-c	1.08 c	0.20 c-e	0.26 g	0.09 jkl
	10	0.46 ij	0.45 hi	0.61 g	0.37 e-i	0.00 h	0.16 g-i	0.14 ij	0.04 p
	20	0.36 k	0.52 gh	0.38 i	0.23 jk	0.00 h	0.13 ij	0.11 jk	0.12 bc
	40	0.43 j	0.11 k	0.00 j	0.17 kl	0.23 gf	0.03 l	0.00 l	0.12 bc
	60	0.56 h	0.63 gf	0.00 j	0.42 d-h	1.20 b	0.17 d-g	0.00 l	0.09 k-m
50	0	0.76 ef	0.80 a-d	0.87 d	0.55 a-c	0.92 c	0.30 a	0.34 cd	0.08 l-n
	10	0.47 ij	0.49 h	0.59 gh	0.36 f-i	0.14 gh	0.17 e-h	0.19 h	0.17 cd
	20	0.65 g	0.71 c-f	0.51 h	0.51 b-d	0.23 fg	0.17 d-g	0.13 ij	0.06 m-o
	40	0.13 n	0.80 a-e	0.00 j	0.47 c-e	0.13 gh	0.19 d-g	0.09 k	0.14 e-g
	60	1.43 b	0.68 ef	1.12 b	0.52 b-d	1.50 a	0.17 d-g	0.27 fg	0.15 de
75	0	0.71 f	0.82 a-c	0.00 j	0.61 ab	0.16 gh	0.20 c-e	0.00 l	0.12 g-i
	10	0.22 ml	0.10 k	0.00 j	0.35 g-i	0.24 fg	0.00 m	0.00 l	0.20 b
	20	0.25 l	0.72 c-e	0.00 j	0.46 c-f	0.17 f-h	0.16 g-i	0.00 l	0.00 q
	40	0.18 mn	0.53 gh	0.00 j	0.41 e-h	0.24 fg	0.21 cd	0.00 l	0.00 q
	60	1.91 a	0.87 ab	1.50 a	0.63 a	0.36 ef	0.29 a	0.43 a	0.24 a
100	0	0.78 e	0.31 j	0.90 d	0.12 l	0.56 d	0.00 m	0.00 l	0.20 b
	10	0.00 o	0.37 ij	0.00 j	0.29 ij	0.60 d	0.14 l-h	0.00 l	0.11 i-k
	20	1.20 d	0.82 a-c	1.28 c	0.59 ab	0.46 de	0.17 e-h	0.30 ef	0.13 e-h
	40	1.33 c	0.92 a	1.41 b	0.63 a	0.09 gh	0.21 c	0.39 b	0.14 ef
	60	0.58 h	0.13 k	0.00 j	0.17 kl	0.00 h	0.00 m	0.00 l	0.14 ef

the third major constituent of essential oil that was affected by the interaction of salinity and humic acid (Table 8). The highest and lowest amounts of this compound were 8.4% and 0.34% in S3H1 and S2H4, respectively (Table 9).

Mean comparison of the interaction between salinity and humic acid for myrcene, p-cymene, and α- thujene showed the same trend in response to application of humic acid under salinity. In fact, under non-saline and 100 mM NaCl, application of humic acid increased the percentage of these EO constituents, but under 25, 50, and 75 mM NaCl conditions, humic acid

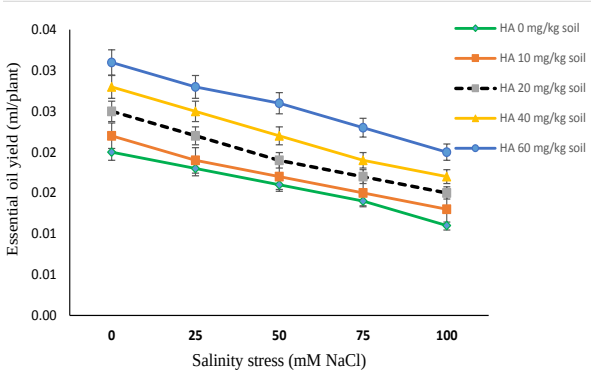

Fig. V. Interaction of salinity and humic acid on essential oil percent in *S. khuzestanica* Jamzad

decreased their percentage (Table 9). The citronellol content was enhanced by humic acid application under non-saline conditions. Meanwhile, its content decreased by humic acid application under salinity stress (25-100 mM NaCl) (Table 9).

The trend of α-pinene showed that it increased by application of humic acid (40 mg/kg soil) under non-salinity and high salinity (100 mM NaCl) conditions. On the other hand, under 25, 50, and 75 mM NaCl salinity conditions, the maximum content of α-pinene was obtained by non-application of humic acid (Table 9). As for α-phellandrene, it increased by application of humic acid (40 mg/kg soil) under 0, 75 and 100 mM NaCl although under 25 and 50 mM NaCl, the maximum content of α-pinene was obtained by non-application of humic acid (Table 9).

Responses of β-bisabolene, limonene, caryophyllene, β-phellandrene, camphene, β-trans-ocimene, and borneol to salinity and humic acid were almost the same. Their maximum contents under non-saline conditions were obtained by humic acid 40 mg/kg soil; however, their maximum contents were obtained under 25 mM NaCl by non-application of humic acid; and under 50, 75, and 100 mM NaCl by application of high level (60 mg/kg) of HA.

Interaction of salinity and HA application was significant for thymol content (Table 8). It hit a maximum content under humic acid treatment 20 mg/kg soil under non-saline conditions, 60 mg/kg soil under 25, 50, and 75 mM NaCl, and 10 mg/kg soil under 100 mM NaCl.

Discussion

Morphological characteristics and dry weight

Anoshee and Farzami Sepehr (2016) have reported decreasing root length of *Lycpoersicum esculemtum* because of salinity. However, this decrease has been mitigated by application of *Glomus mosseae*. Humic acid improved root length noticeably. However, using a high concentration of humic acid (60 mg/kg soil) is not recommended because it did not result in a significant difference from 40 mg/kg soil. Mitigation of adverse effects of salt stress in summer savory (*S. hortensis* L.) by biochar has been reported (Mehdizadeh et al., 2019). About *S. khuzestanica*, it has been reported that soil application of humic acid under salinity stress improved soil physicochemical and biological characteristics (Zaremanesh et al., 2020) so that, increasing secondary branches and leaves may partly be due to the role of humic acid in mitigation of salinity effect on soil. Meanwhile, the other functions of humic acid, e.g. as a stimulant for bacterial growth, nutrient, organic acid, and amino acid supplier (Khattak et al., 2013) and alkaline soil pH adjustment (Karakut et al., 2009) must not be ignored.

Vojodi Mehrabani et al., (2017) also reported a decrease in the dry weight due to salinity, so that the lowest dry weights of roots, stems, and leaves were observed under the highest salinity level (150 mM NaCl). It has been reported that humic acid mitigated the salinity effects on savory plants and improved growth parameters under greenhouse conditions (Khosropour et al., 2015). Also, Kaya et al. (2018) reported that using humic acid (100 mM) in maize under salinity stress significantly increased the plant biomass, Fv/Fm, chlorophyll pigments, and proline contents, but reduced the H_2O_2 and MDA contents.

By increasing the concentration of salts, water and nutrient uptake by the plant decreases due to increasing the osmotic potential in soil. Salinity leads to a decrease in plant growth rate and vegetative characteristics, which finally leads to decrease in the dry weight. Other reasons for the reduced dry weight of the plants under salinity stress are related to decreased photosynthesis due to reduced leaf area, reduced stomatal conduction, accumulation of chlorine and sodium in the organs, or destruction of chloroplasts (Taiz et al., 2015).

Physiological characteristics

Decrease in chlorophyll content by salinity is an indication of oxidative stress, attributed to the inhibition of chlorophyll synthesis, and the activation of its degradation by the enzyme chlorophyllase (Santos, 2004). Besides, chlorophyll reduction has been reported under salinity stress due to nitrogen consumption in the synthesis of compounds such as proline. Proline

plays key roles against stress by acting as a compatible solute, conserving cytoplasmic enzymes, scavenging free radicals, and preventing damage to the cell membrane (Sivritepe et al., 2010).

Salinity stress produces ROS that destroys chlorophyll and chloroplasts. Under salinity conditions, the uptake of ions such as magnesium and iron, which play a vital role in the structure of chloroplasts, is impaired, reducing the synthesis of chlorophyll and resulting in reduced photosynthesis. Applying humic acid can reduce the effect of stress on chlorophyll synthesis. The use of humic acid in roses has increased the amount of chlorophyll a and b. The highest amount of chlorophylls was observed in 1000 mg/l humic acid as soil treatment. However, its excessive use (2000 mg/l), decreased the chlorophyll content (Jabbarzadeh and Talebi, 2018). Increased leaf chlorophyll content may be due to an improved nitrogen uptake, nitrogen metabolism, and the production of protective proteins as a result of applying humic acid (Haghighi et al., 2012). Nitrogen has an essential role in increasing plant chlorophyll. Due to the increase in nitrogen uptake in the presence of humic acid, it may have improved the absorption of nutrients and subsequently increased the chlorophyll content.

It has been reported that carotenoids content decreased with increasing salinity even in halophyte plants such as *Suaeda* and *Salicornia* (Akcin and Yalcin, 2016). Carotenoids are soluble antioxidant compounds in plant cells which reduce the oxidative damages in non-enzymatic manner. They are present in the chloroplasts and under the environmentally induced oxidative stress they are responsible for protecting photosynthetic apparatus, particularly chlorophylls, against the ROS (Rahal et al., 2014).

Yang et al. (2004) reported that humic acid increased flavonoids contents of *Ginko biloba*. Flavonoids are secondary metabolites and have vital roles in resistance against oxidative damage (Pandey et al., 2017). Accumulation of flavonoids, such as flavonols, anthocyanins, and proanthocyanidins in plants could enhance salt tolerance (Chen et al., 2019). Therefore, moderate salt stress could increase flavonoids and improve the quality of crops and medicinal plants (Colla et al., 2013). A study on the effect of salt stress on safflower showed that when salinity level reached 50 mM NaCl, the SOD activity increased in all genotypes (Shahbazi and Golkar, 2017). Based on the results of Narimani et al. (2018) on *Deracocephalum*, when salinity reached 100 and 150 mM NaCl, the application of humic acid (200 mg/l) resulted in a significant increase in the activity of ascorbate peroxidase and CAT. Thus, under high stress levels the role of humic acid was completely noticeable as a stress mitigation compound. However, in maize, exogenous application of humic acid (100 mM) as foliar or seed treatment reduced H_2O_2 and MDA contents as well as SOD, CAT, and POD activities (Kaya et al., 2018).

Positive effects of humic acid on promoting plant growth have been reported in many studies (Ouni et al., 2014; Quaggiotti et al., 2004; Sánchez-Sánchez et al., 2006). Part of this growth promotion is mediated by ROS production. It has been reported that growth caused by the application of HA was mediated by ROS production. The increase in ROS production may induce the expression of antioxidant genes such as catalase to decrease the oxidative effects of the ROS necessary for growth (Cordeiro et al., 2011)

Increasing POD activity by salinity has been reported by other researchers (Haddadi et al., 2016; Kaya et al., 2018; Yildiztekin et al., 2018). Humic acid mitigated salinity effect, so that it reduced POD activity. Sebastiano (2005) reported that humic acid increased kinases, phosphatases, and cytochrome oxidase but inhibited indole acetic acid oxidase and peroxidases. These findings are in line with our results. As peroxidase activity increases under stress and the rate of increase is higher in resistant species, the chlorophyll stability index is greater and the damage to their cell membranes is less. Therefore, it seems that when salinity stress occurs, the increased activity of peroxidases, as much as possible, prevents the harmful effects of various reactive oxygen species on the cell membrane (Pandey et al., 2017).

In each level of salinity, the application of humic acid increased GR activity. It has been reported

that the GR activity increased in maize (AbdElgawad et al., 2016) and lentil (Gaafar and Seyam, 2018) roots by salinity stress. Antioxidant enzymes are the most essential components of the ROS scavenging pathway. In this pathway, GR plays a significant role in plant adaptation to oxidative stress. This enzyme reduced GSSG to GSH. High GSH/GSSG ratio is useful in detoxifying H_2O_2 (Taïbi et al., 2016).

Alizadeh et al., (2016) reported that the highest percentage of EO (3.33) of *S. khuzestanica* was obtained from the application of 1.5 l/h of humic acid and 20% of vermicompost extract. However, the highest EO yield (6.38 g/m2) was produced by 3.5 l/h humic acid and 20% vermicompost extract. Application of vermicompost increased leaf number, fresh weight, and plant height in *S. hortensis* (Naiji and Souri, 2015). It has been reported that biofertilizers and vermicompost had significant effects ($p \leq 0.01$) on biological yield and essential oil contents of leaves in *S. hortensis* (Rezvani Moghaddam et al., 2013). Thus, it seems that in our research, humic acid as an organic fertilizer has improved growth index that resulted in EO yield.

This reduction in the EO content may be due to the reduction of photosynthesis because of salinity (Zaremanesh et al., 2019) which eventually leads to less biomass production. Organic fertilizers increase the synthesis of essential oils by providing nutrients, especially micronutrients (Alizadeh et al., 2016). Humic acid increases the amount of EO by enhancing absorption of phosphorus and nitrogen, which are present in the components of the EO. Nitrogen also provides a suitable base for receiving solar energy by increasing the area and number of leaves and by participating in the structure of chlorophyll and enzymes involved in photosynthetic carbon metabolism; it improves photosynthetic efficiency and plays a crucial role in increasing the amount of EO (Niakan et al., 2004).

Estaji et al. (2018) have identified 25 compounds in the oil obtained from *S. hortensis* under salinity stress, where the majority of these compounds were carvacrol (19–46.6%), γ-terpinene (11.59–24.8%), p-cymene (9.84–34.56%), myrcene (1.4–2.78%), and β-pinene (1.20–1.91%). Khadivi-Khub et al. (2014) reported thirty-four components in the EO of S. bachtiarica among which carvacrol, thymol, gamma-terpinene, and p-cymene were the major ones.

As described above, the interaction of treatments on EO compounds revealed that essential oils change as secondary metabolites under the influence of environmental and nutritional conditions. Shahnazi et al. (2008) identified 34 compounds in *S. intermedia* essential oil, the most important of which were hydrocarbon monoterpenes, including thymol, paracemen, gamma terpinene, carvacrol, alpha terpinene, and myrcene). The main constituents of *S. sahandica* essential oil has been reported as thymol, paracemen, and gamma terpinin by Sefidkon et al. (2004). In another study, about 99.3-96.4 of the total composition of *S. khuzestanica* EO included nine compounds of carvacrol, gamma terpinin, alpha thujene, myrcene, alpha terpinene, paracemen, thymol, eugenol, and alpha-pinen (Nooshkam et al., 2015).

Savory species have a great variety in terms of the amount and type of compounds. In the EO of some species, the major constituents are polygene and menthol while in the essential oil of some other species compounds such as carvacrol, gamma-terpinene, and parimene constitute the major constituents of the essential oil (Ahmadi et al., 2009).

In an experiment on garden savory collected from 15 countries, it was found that in addition to differences in morphology, many changes were observed in the quantity and quality of EO. Therefore, different environmental conditions can make a big difference in the quantity and quality of essential oils (Yu et al., 2015).

Therefore, the results obtained in the current research are somewhat consistent with the results of other studies. Differences in the amount of some of the constituents in the EO can be related to the environmental conditions, type of nutrition, or harvest time.

Conclusion

With increasing salinity stress, growth and physiological traits (dry weight, root length, number of leaves, plant height, and contents of chlorophyll and carotenoids), the percentage, and yield of EO decreased. However, flavonoids content and antioxidant enzymes activity increased. Application of humic acid reduced the effect of salinity on these traits. It seems that humic acid has increased plant performance by reducing the effect of salinity, improving the supply of plant nutrients, increasing water holding capacity, and improving the activity of beneficial soil microorganisms.

The major constituents of EO were increased by using humic acid in saline conditions; however, the rest, which featured a low percentage of the total EO decreased. This may be related to the plant's strategy for coping with stress. This issue can be both a useful guide for producers of EO in saline conditions and considered as a research topic for those interested in stress physiology in *S. khuzestanica*.

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