 doi:10.20944/preprints202108.0059.v1

Effect of salinity on morphological characteristics, biochemical accumulation, and essential oil yield and composition in *Black horehound (Ballota nigra L.)*

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Abstract: Black horehound *(Ballota nigra* L.) is one of the important medicinal plants, which is a rich source of health-promoting essential oils. Salinity stress affects plant development and alters the quality and quantity of plants extracts and their composition. This study was aimed to investigate the effect of salinity on morphological, physiological characteristics, and secondary metabolites of *B. nigra* under greenhouse, and *in vitro* culture conditions. The plants were treated with different concentrations of NaCl (25, 50, 75, 100 mM) and fresh and dry weight of leaf and stem were measured as well as morphological characteristics of the plant. Plant growth was reduced with the increased salinity concentrations. The results showed that all growth-related traits and SPAD were decreased both *in vivo* and *in vitro*. Additionally, increased salt concentration affected the cell membrane integrity. Total phenolics content of plants growing in the greenhouse, increased by 21% at 50 mM NaCl, but at higher stress levels (100 mM NaCl), the amounts were decreased significantly. Total flavonoids contents followed similar patterns, with a slight difference. In addition, the maximum and minimum total phenolics contents of plants growing under *in vitro* condition were observed at 50 mM NaCl and control treatments, respectively. Increasing the salt concentration significantly affected the total flavonoids content, and as a result, the highest amount was observed in 50 and 75 mM NaCl treatments. Antioxidant activity was also measured. Among the NaCl treatments, the highest DPPH scavenging activities (IC50) under greenhouse and *in vitro* conditions were detected at 50 mM and 25 mM concentrations, respectively. In general, based on the results, with increasing the salinity level to 75 mM, the activities of CAT and APX were significantly upregulated in both greenhouse and *in vitro* culture conditions. A correlation between total phenolics and flavonoids contents as well as antioxidant activity were obtained. With shifting salinity stress, the type and the amount of the identified essential oil compounds changed. Compounds such as styrene, tridecanol, germacrene-D, beta-Ionone, beta-bisabolene, and caryophyllene oxide increased compared to the controlled treatment.

Keywords: *Ballota nigra, salinity, antioxidant, enzyme activity, phytochemical composition*

Introduction

Soil salinity is considered one of the important abiotic factors significantly limits the growth of plants resulting decrease in the agricultural crop production and sustainability worldwide. To-date, nearly 20 % of the world's agricultural land is under the saline condition, impacting on severe losses estimated to 12 billion USD each year (Arzani, 2008; Flowers et al 2010). It is predicted that the soil salinity to increase in the wake of climate change (Xie et al.

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2017). The soil salinity is worse and more prevalent in arid and semi-arid regions where the frequent drought occurs, causing high evaporation, leading to accumulation of various salts in the surface layers of the soil (Fathizad et al., 2020). Thus, salinity exposes plants to secondary drought stress (Sairam et al., 2002).

Ballota nigra L. is a perennial medicinal plant belongs to mint family, Lamiaceae. It is widely distributed in temperate and subtropical regions in Europe and the Middle East. The plant inhabits waste places and roadsides in human settlements, as well as weedy places and cultivated grounds (Popova, 1979). Species of the genus *Ballota* L. have been traditionally used for cardiovascular diseases and inflammation of the respiratory tract, and other medicinal purposes to cure nausea, vomiting, nervous dyspepsia, as well as sedative, mild astringent, and antimicrobial agents (Sahin et al., 2005). About 20% US population use herbal medicine supplements (Kennedy, 2005). However, the quality and quantity of these herbal metabolites totally depend on the environmental conditions such as soil salinity and drought.

High concentrations of salt in the soil can have devastating and significant effects on various aspects of plant growth and development, including germination, yield, nutrient uptake, photosynthesis, growth, quality and quantity of primary and secondary metabolites. Increasing the amount of sodium in cellular organs causes the destruction of the embryo in seeds, membranes and hypocotyls of seedlings (Khan et al., 2000). Salt stress affects medicinal plants on different physiological stages, including seed germination, seedling growth by blocking the mobilization of reserve foods, injuring hypocotyls, and suspending cell division (Belaqziz et al., 2009; Said-Al Ahl and Omer, 2011; Vafadar et al., 2020). Morphological characteristics such as leaf number, leaf area, and biomass have been reduced under salt stress in a number of medicinal plants, *Thymus vulgaris* (Najafian et al., 2009) and *Mentha pulegium* (Queslati et al., 2010).

Salinity stress leads to oxidative stress in plants with an increase in reactive oxygen species (Zhu, 2001). Radicals resulting from incomplete oxygen, reduce target precursor biomolecules of metabolites that are essential for metabolism and inhibit their synthesis. In addition, by invading vital cell macromolecules such as, nucleic acids, proteins, and lipids, can cause mutations in the structure of DNA, alter the nature of proteins, and damage membranes, respectively (Amanifar et al., 2019; Farsaraei et al., 2020; Jaiswal et al., 2020). Reports show that the proline content as a stress osmolyte increases under salinity stress in plants such as, peppermint, sage, and summer savory (Banerjee and Roychoudhury, 2018). The increase in proline content is related to the decrease in the activity of key enzyme proline oxidase, which ultimately maintains the osmotic balance in the cells and continuous absorption of water under salinity stress. Salinity stress affects the production of secondary metabolites and essential oils in medicinal plants (Selmar, 2008; Bistgani et al., 2019; Vafadar et al., 2020).

The composition of essential oil, the content of phenolic compounds, proline, photosynthetic pigments and percentage of essential oil are severely altered in the plants grown under salinity conditions (Vafadar et al., 2020). The reduced yield of essential oil under salinity stress was also recorded in ajowan, basil, fennel, and thyme (Banerjee and Roychoudhury, 2018). Furthermore, the constituents of essential oils are also affected by salinity stress. The levels of terpenoids such as menthol in mint, carvacrol in coriander, 1, 8-cineole in sage, linalool in basil increase with increasing salinity, and in contrast, the level of compounds such as gamma terpenene in coriander, p-cymen in marjoram, and eugenol were reduced in basil (Aziz et al., 2008; Neffati and Marzouk, 2008; Said-Al Ahl et al., 2010). Although the effects of salt stress on crops have been widely studied, very little information is available on the impact of salt stress on primary and secondary metabolites and regulation of antioxidative defense in medicinal plants.

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Salt stress significantly affects the production of essential oils and the constituents of medicinal plants, thus the investigation on the mechanisms of salt tolerance in medicinal plants has a great importance. Due to the importance of fragrant species in terms of medicine, more researches are necessary to explore the production of essential oils and other secondary metabolites and the growth of these plants under salt stress. Creation of salt-tolerant medicinal plant leads to increased production of raw materials for drugs, flavors, fragrances, and spices all over the world. In order to increase the production of a special compound in a medicinal plant it is necessary to know the distribution of the metabolites in the plants in response to various levels of salt stress. Hence, the present study was aimed to investigate the effects of salinity stress of sodium chloride on morphological, physiological characteristics and secondary metabolites of *B. nigra in vivo* and *in vitro* culture conditions.

Results and discussion

Soil salinity inhibited plant growth and yield: Salinity has caused significant reduction in plant growth. The results studied on plant morphological traits of leaf number, leaf area, root and shoot length, fresh and dry weights of both root and shoot tissues, SPAD and branch numbers showed significant reduction by salt treatment. Analysis of variance data showed that salinity stress has a significant effect ($P<0.01$) on all growth-related traits both *in vivo* and *in vitro* (Table 1). The values observed for leaf number, leaf area, root length, root fresh/dry weight, shoot length, shoot fresh/dry weight, SPAD, and branch number were gradually reduced from control to NaCl treated plants from 25mM to 100 mM (Table 2).

Table 1. ANOVA of salinity stress effect on growth parameter measured in greenhouse and *in vitro* conditions

		Mean of squares (greenhouse)									Mean of squares (in vitro)			
S.V	d.f	Leaf Number	Leaf Area	Root Length	Root Fresh Weight	Root Dry Weight	Shoot Length	Shoot Fresh Weight	Shoot Dry Weight	SPAD	d.f	Leaf Number	Shoot Length	Number of Branch
Repeat	2	45.3	1431.9	0.3	40.7	0.8	0.1	158.5	7.05	9.2	-	-	-	-
Salinity	4	52.7**	6690.9**	20.4**	519.8**	6.0**	20.4**	47.0**	12.2**	106.2**	4	729.1**	4.5**	19.7**
Error	8	21.0	257.1	0.4	8.8	0.2	0.4	24.32	0.7	1.4	10	4.3	0.3	0.6
C.V		10.5	3.8	2.1	8.1	5.7	2.1	8.2	6.9	3.7		10.2	18.7	20.6

Table 2. The effect of salinity stress on growth parameter measured in greenhouse and *in vitro* conditions

		NaCl (mM)				
Condition	Characteristics	0	25	50	75	100
Greenhouse	Leaf Number	66.5 ± 2.2 *a	48.3 ± 2.1 b	37.2 ± 1.2 c	33 ± 1.2 c	32 ± 0.8 c
	Leaf Area	476.3 ± 7.2 a	446 ± 14.5 a	396.5 ± 8.5 b	372.5 ± 10.2 c	351.2 ± 15.6 c
	Root Length	33.2 ± 1.2 a	33.6 ± 1.5 a	29.3 ± 0.8 b	28.3 ± 0.4 b	28.2 ± 0.3 b
	Root Fresh Weight	52.9 ± 1.9 a	46.5 ± 1.6 b	34.8 ± 2.5 c	27.7 ± 1.2 d	20.8 ± 1.1 e
	Root Dry Weight	9.2 ± 0.4 a	8.1 ± 0.3 b	6.6 ± 0.4 c	6.2 ± 0.4 c	5.9 ±0. 5 c
	Shoot Length	31.5 ±0.5 a	28.6 ±0.6 b	24.2 ±0.6 c	23.2 ±0.4 c	21.3 ±0.3 d
	Shoot Fresh Weight	78. ±1.1 a	64.2±3.6 b	60.8±3.5 bc	49.5 ±2.2 cd	48±2.8 d
	Shoot dry Weight	15.2±1.5 a	12.9±1.4 b	12.2±1.8 bc	11.2±1.4 cd	9.8±1.3 d
	SPAD	40.4±1.5a	35.3±0.3b	33.5±0.3b	29.3±0.2c	24.7±1.2d
In vitro	Leaf Number	45± 0.6 a	23.6 ± 1.1 b	18 ± 1.2 c	12 ± 1.1 d	3.7 ± 0.7 e
	Shoot Length	4.6 ± 0.3 a	3.6 ± 0.6 ab	2.7 ± 0.5 bc	2.5 ± 0.4 c	1.4 ± 0.5 d
	Number of branch	7.8 ± 0.7 a	4 ± 0.5 b	3 ± 0.5 b	3 ± 0.5 b	1 ± 0.3 d

* Means along with the same letter are not significantly different at 5% significantly level ($P \leq 0.05$)

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Leaf area/number decreased after the plant was exposed to higher concentration of NaCl solution. Exposure to higher NaCl levels caused severe damage to photosynthetic tissues and reduction in leaf gas exchange due to ion concentration in leaf tissues. Furthermore, there was a greater reduction in turgor pressure and water potential required for cell development, which have caused reduction the leaf area in plants grown *in vivo* and *in vitro*. Plants with reduced leaf area exhibit low evaporation rate and prevents water loss resulting in reduction in photosynthetic material (Gebauer et al., 2004). Overall, impaired absorption of important ions and elements such as calcium and nitrogen, and closed stomata to prevent water loss reduce water uptake from the soil affecting the plant growth (Cramer, 2002; Bhatt and Rao, 2005). Previous results have also shown that shoot length fresh weight of *B. nigra* decreases significantly due to increasing salt concentration (Rezgui et al., 2017).

SPAD value was reduced by increasing the NaCl concentration (Table 2). Reduction in SPAD value and photosynthetic pigments under salt stress induced reduction in catalytic activity of the amino levulinic acid synthase enzyme, which catalyze the production of 5-aminolevulinic acid; a key precursor in chlorophyll pigment biosynthesis pathway. Furthermore, increasing of chlorophyllase activity, photoinhibition, and accumulation of reactive oxygen species (ROS) under salinity stress resulted in degradation of photosynthetic pigments. Similar reduction in growth indices of medicinal plants have been earlier reported in basil, *Salvia spp.*, *Carthamus* and *Dracocephalum* (Taarit et al., 2009; Heidari and Golpayegani, 2012; Harrathi et al., 2013; Vafadar et al., 2020).

Effect of salinity on cell membrane integrity in greenhouse grown plants The cell membrane stability was severed in the plants treated with higher saline conditions. The results showed that NaCl concentration at 50mM and above showed an increase in electrolyte leakage compared to the control plants (Figure 1). Higher salt concentrations cause is damage to the plasma membrane through production and accumulation of ions (K^+/Na^+) and reactive oxygen species, such as superoxide radicals, hydrogen peroxide and hydroxyl radicals causing severe damage to plasma membrane (Bistgani et al., 2019). These ions due to their high level of energy and reactivity damage many cellular compounds such as fats, proteins, carbohydrates, and nucleic acids, and by altering membrane structure of fats and proteins, causing electrolyte leakage (Demidchik et al., 2014).

Effect of salinity on total phenols, flavonoids, and antioxidant enzyme activities:

Plant secondary metabolites such as, phenol and total flavonoids have strong potential for scavenging free radicals (Mathew and Abraham, 2006). These compounds act as electron donors and may neutralize unwanted reactions caused by free radicals in living organisms (Manian et al., 2008). Presence of total phenolic compounds often correlate to the antioxidant activity of the plant products. In this study, the total phenol contents were found in higher amounts *in vitro* grown plants when compared to plants *in vivo*. These results were similar to the earlier reports observed in vitro cultures of *B. nigra (*Makowczynska et al., 2015*)*. Total phenol contents were increase by 20% in response to moderate salt stress (up to 50 mM NaCl), and starts declining at higher salinity levels (75 and 100 mM NaCl) in both *in vivo* and *in vitro* plants (Figure 1). Peppermint and rosemary extract have shown high antioxidant activity which is directly related to higher phenolic content of the plants (Kanatt et al., 2007). Higher concentrations of total flavonoids were detected at in vivo grown plants compared to *in vitro*. However, their values were significantly reduced to high salinity treatments in plants grown under both environments. Earlier studies on high concentration of NaCl induced saline treatments showed similar reduction on the on total phenol and flavonoid contents (Zhou et al., 2018; Vafadar et al., 2020).

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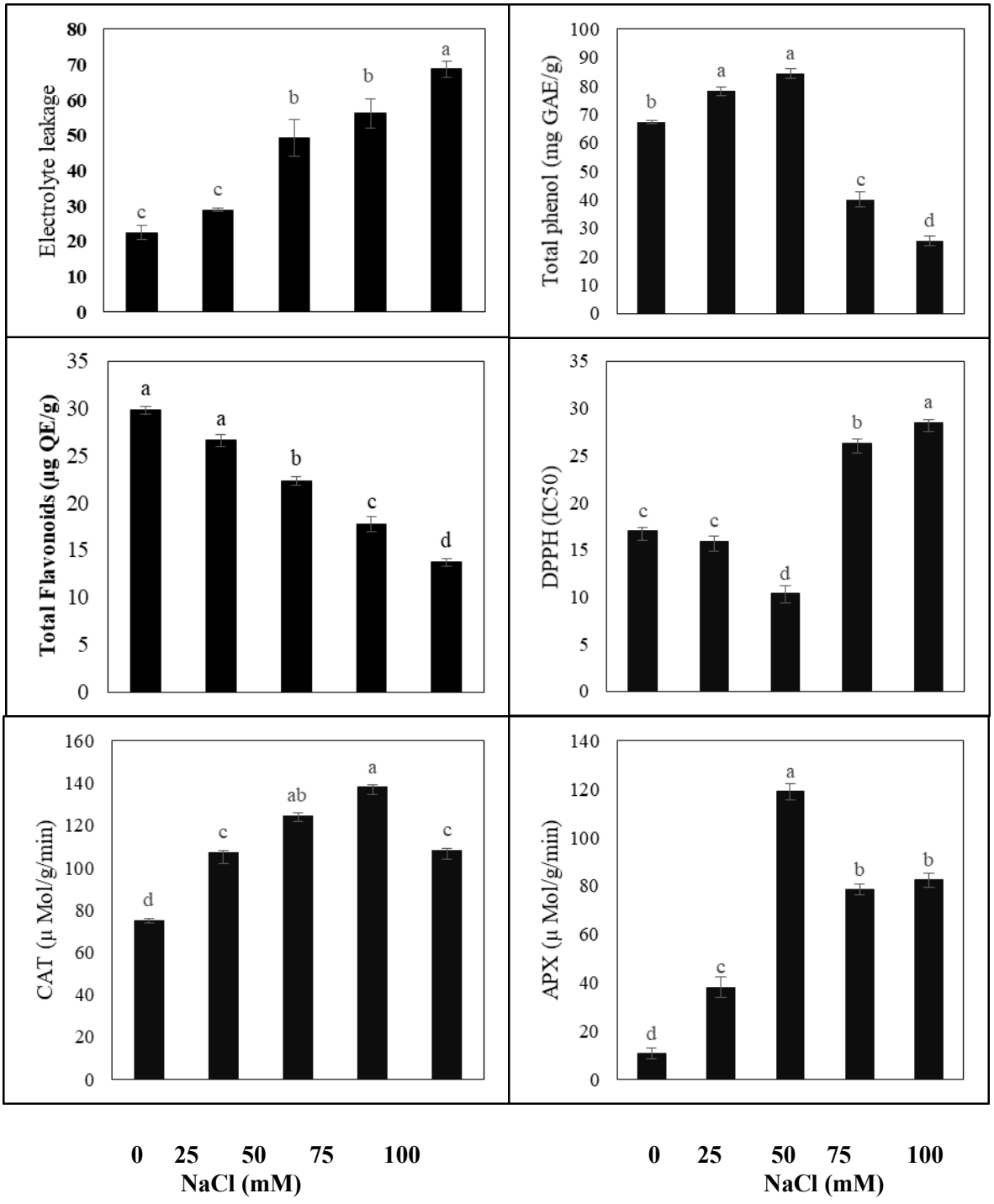

Figure 1. The effect of salinity stress on physiological related parameter measured in greenhouse.

Antioxidant activity: CAT and APX enzymes are the important antioxidants, which improve plant defense system to deal with the abiotic stresses. These enzymes can directly convert hydrogen peroxide to water and oxygen and completely eliminate the toxicity of this oxygen free radical (Gill and Tuteja, 2010).

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Our results indicate that CAT levels were higher *in vivo* than *in vitro*. The levels of CAT were increased up to 75 mM *in vivo*, while their levels increased up to 50 mM *in vitro*. APX was found in similar levels in both *in vivo* and *in vitro* and their levels were elevated to moderate levels of salinity stress (50 and 75 mM NaCl respectively) under both conditions. For both CAT and APX, NaCl at 100 mM found to be detrimental as the activity of both enzymes reduced significantly to high salt stress. It must be noted that, both APX and CAT are both enzymatic components of antioxidant machinery with different affinities for hydrogen peroxide, where in, APX is responsible for fine tuning of reactive oxygen species for signaling and more efficient scavenger of H2O2, while CAT removes excess ROS under stress (Mittler, 2002). In this experiment, the equal amounts of APX in both plant tissues and *in vitro* cultures suggests its role in regulating ROS for signaling at high salt stress conditions (NaCl >75 mM). The APX is shown to increase in salt tolerant cultivars while it significantly diminishes in salt susceptible cultivars (Aghaei et al., 2009). APX is widely spread in cell organelles detoxify H_2O_2 induced in response to environmental stresses (Das and Roychoiudhury, 2014). Several transcription factors are reported to regulate APX genes mediated by Ca^{2+}-regulated expression of *APX* genes and improves plant tolerance against waterlogging stress (Liu et al., 2012; Yan et al., 2015). The decrease in activity level of these enzymes at high salt concentration (100 mM) may be due to the increase in compounds such as hydrogen peroxide, which indicates that this level of salinity stress is beyond the tolerance of *B. nigra* plant. At higher salinity environment, the plant will not be able to modulate the oxidative stress caused by ROS, and consequently electrolyte leakage, membrane disintegration and photosynthetic pigment depredation will be intensified at this stress level (Vafadar et al., 2020).

The antioxidant enzymes work in conjunction to alleviate the damaging effects of ROS and develop tolerance to stress. However, the signaling and regulation of ROS to salt stress is still not clearly understood. Its hypothesized that, calcium signaling paly role in regulating ROS. The expression and activities of antioxidant enzymes are controlled both directly and indirectly at multiple levels with the involvement of ubiquitous secondary messengers (ROS, RNS, and Ca^{2+}), PTMs (phosphorylation and redox-dependent ones), TFs, and other precise mechanisms (Dvorak et al., 2021). Further systems biology approach will offer detailed insights into ROS network and its related responses.

The activity of DPPH at IC50 was measured to assess the antioxidant capacity, which represents the amount of *B. nigra* methanolic extract that (mg) is able to remove by 50% of the DPPH radical in the environment. Among the NaCl treatments, the highest DPPH scavenging activity (IC50) *in vivo* and *in vitro* conditions was detected in 50 mM and 25 mM concentrations, respectively (Figure 2). However, under *in vitro* condition, 25 and 50 mM NaCl treatments did not show significant difference. The strongest DPPH scavenging activity (IC50) was observed (both in greenhouse and in vitro conditions) at concentrations of 25 and 50 mM NaCl treatments, in which the amount of phenolic and flavonoid compounds as antioxidant compounds were maximum (Figures 1 and 2). The lowest IC50 levels were 10.4 (in 50 mM NaCl) and 11.2 (in 25 mM NaCl) mg of methanolic extract under greenhouse and *in vitro* conditions, respectively.

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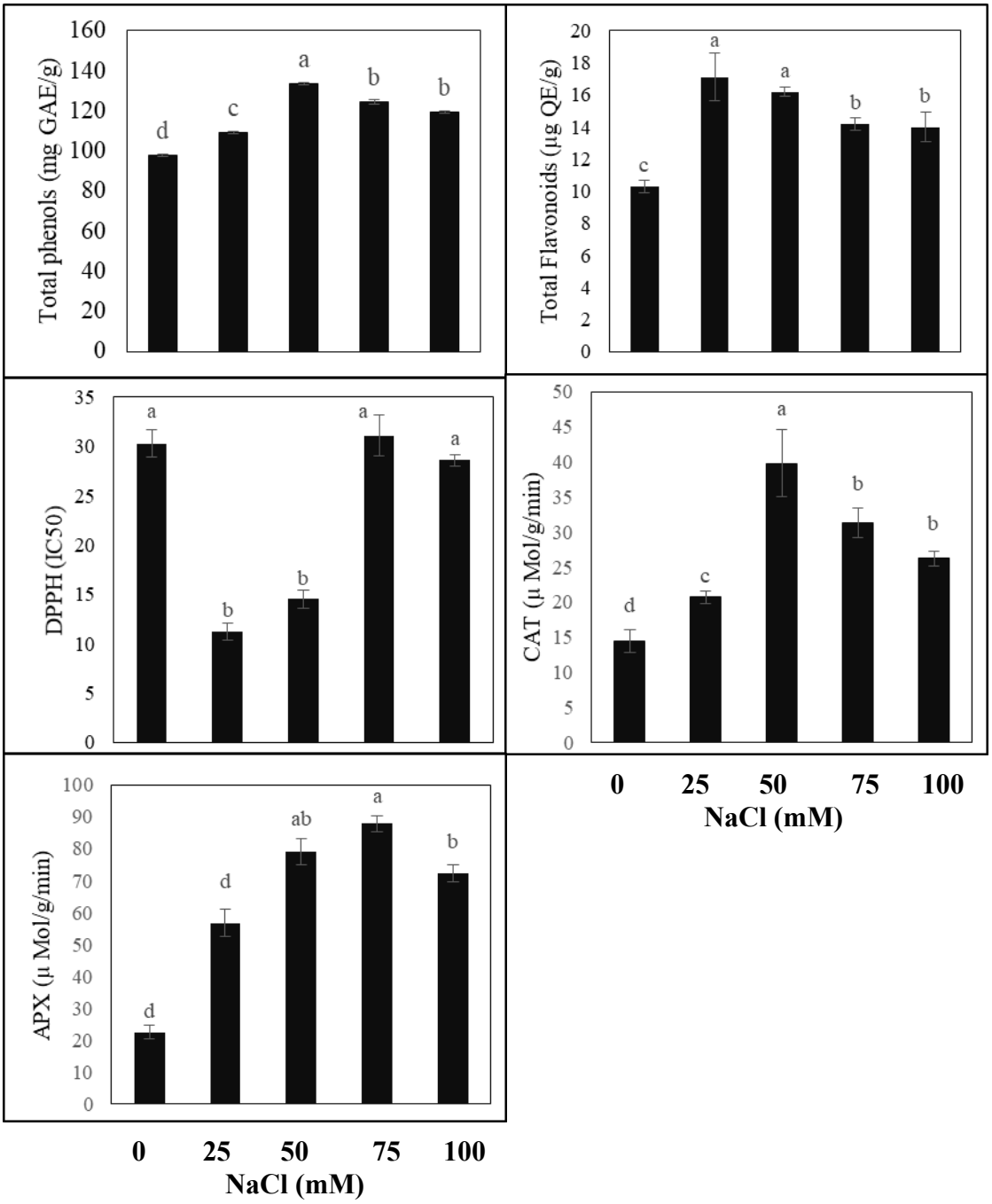

Figure 2. The effect of salinity stress on physiological related parameter measured in in vitro culture

ANOVA results showed that total phenol/flavonoid content, DPPH (IC50) activity, APX and CAT activity were significantly affected ($P<0.01$) by NaCl stress in both growth conditions (*i.e.* greenhouse and *in vitro*) (Table 3). Results showed that the first two principle components PC1 and PC2 explained 83.6 and 12.3% of the whole variance, respectively (Table 4). According to eigen vectors of all variables in each two principle components, the correlation between the variables can be deduced from the biplot (Figure 4). Correlation of CAT and APX enzymes was positive, whereas for and total phenol content and DDPH (IC50) it was negative, respectively.

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Based on the direction of the variables, vectors and treatment points, it can be inferred that the activity of two enzymes and the total phenol content (TPC) reached their maximum value at 50 mM NaCl treatment. On the other hand, DPPH scavenging activity (IC50) and membrane integrity (EL: electrolyte leakage) significantly decreases in higher salinity treatments (i.e., 75 and 100 mM). Overall, these findings are corroborating with the results obtained by mean comparison analysis. The minor change observed is due to the use of only two components which they explained not all but the large portion of variability (95.9% of whole variation).

Table 3. ANOVA of salinity stress effect on physiological related parameter measured in greenhouse and *in vitro* conditions

			Mean of squares					
	Source of variation	d.f	TPh	Flv	DPPH	APX	CAT	EL
Greenhouse	Repeat	2	4.5	0.2	0.5	2391.3	0.8	4.63
	Salinity	4	1916.5**	126.5**	171.2**	132662.0**	1685.1**	1104.0**
	Error	8	28.7	27.8	0.6	4178.9	335.3	54.8
	C.V%		9.1	4.1	4.1	19.6	16.5	16.4
In vitro	Salinity	4	563.6*	20.7**	245.9**	1965.1**	284.4**	-
	Error	8	119.6	2.8	5.6	63.0	46.9	-
	C.V		9.3	11.6	10	12.5	25.8	-

Table 4- PCA based on the variables measured in greenhouse condition under different NaCl treatments

Variables	Principle components	
	PC1	PC 2
Leaf Number	0.27	-0.18
Leaf Area	0.28	-0.01
Root Length	0.26	-0.06
Root Fresh Weight	0.28	0.04
Root Dry Weight	0.28	-0.12
Shoot Length	0.28	-0.08
Shoot Fresh Weight	0.28	0.01
Shoot Dry Weight	0.27	0.03
SPAD	0.27	0.12
Electrolyte leakage	-0.28	-0.03
Total phenols (mg GAE/g)	0.20	0.52
Total Flavonoids (µg QE/g)	0.28	0.11
DPPH (IC50)	-0.18	-0.55
CAT (µ Mol/g/min)	-0.21	0.36
APX (µ Mol/g/min)	-0.21	0.45
Eigen values	12.5	1.8
% of variance	83.6	12.3
Cumulative %	83.6	95.9

Salinity effect on EO yield and quantity: Salinity stress significantly affected the essential oil yield and the composition. Due to the very low yield of the essential oil in *B. nigra* and the difficulty of its analysis in this species, n-hexane was used as a solvent to extract volatiles in this species. Although n-hexane solvent was used to extract volatile oils *in vitro* cultures, it did not yield the compound analysis in GC-MS. Therefore, only greenhouse cultivated *B. nigra* compounds were compared at different NaCl levels, using GC-MS analysis. In total, 39 different

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compounds were detected in the n-hexane extract of *B. nigra* in control as well as all treatments (Figure 3).

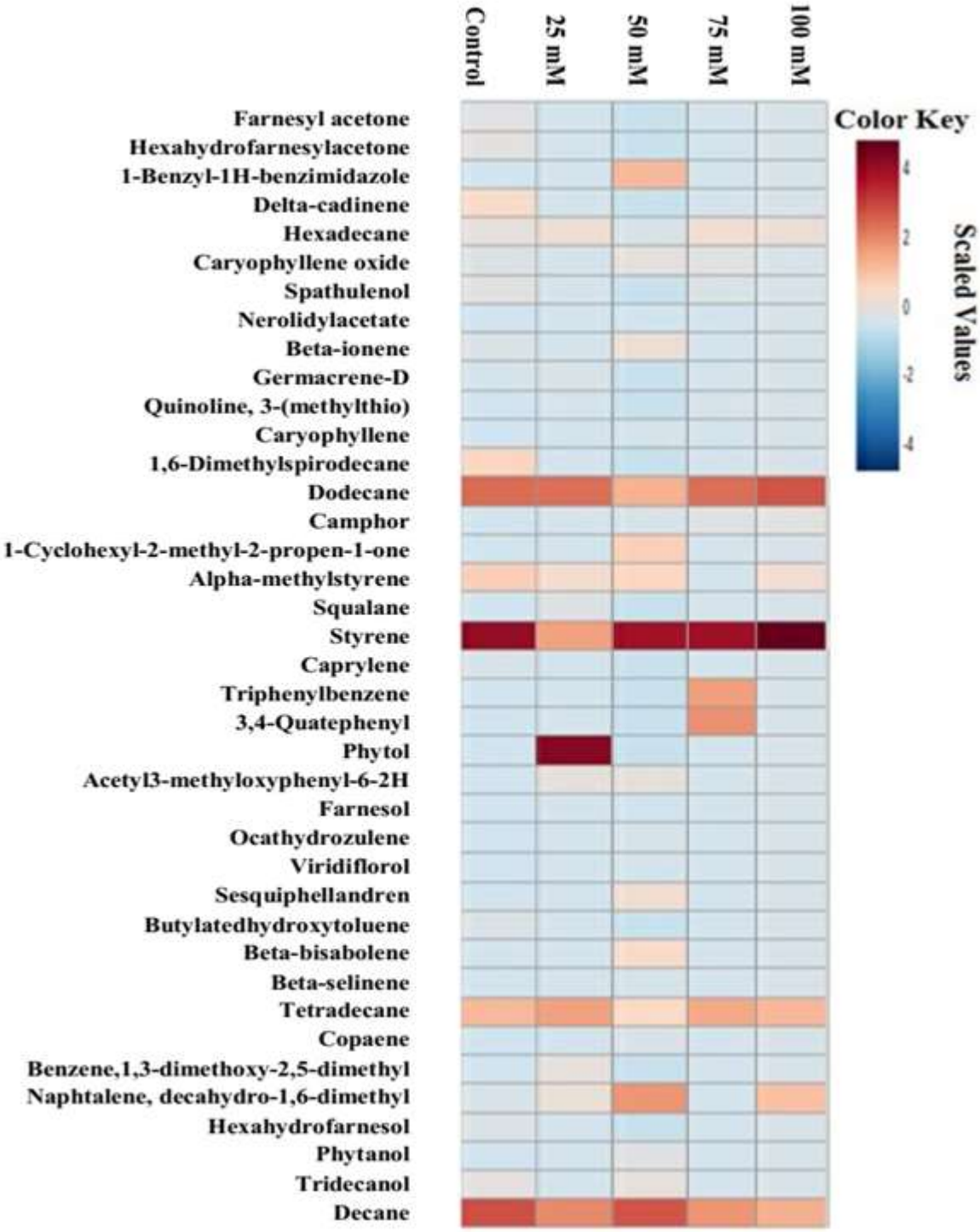

Figure 3. The heat map of detected compounds in B. nigra plants under different NaCl concentrations

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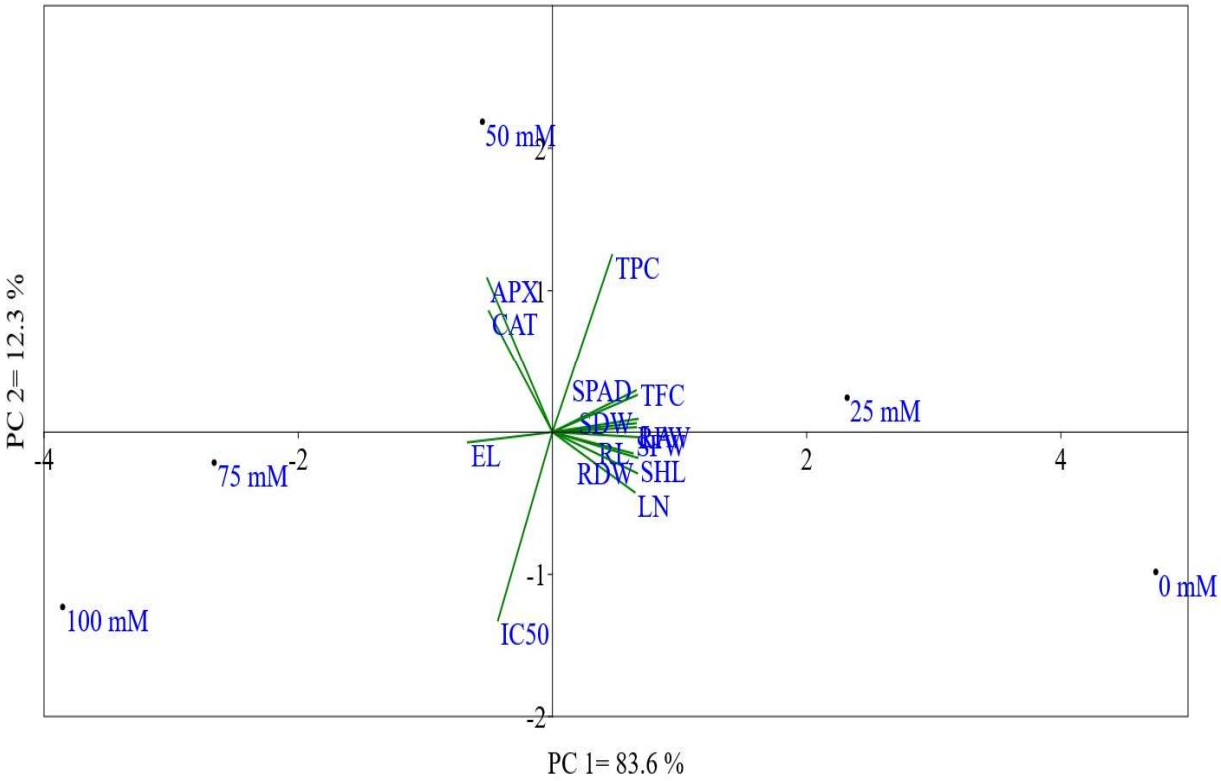

Figure 4. Biplot resulted from PCA procedure for all the variables measured in greenhouse condition under different NaCl treatments (0, 25, 50, 75, and 100 mM). Leaf Number (LA), Leaf Area (LA), Root Length (RL), Root Fresh Weight (RFW), Root Dry Weight (RDW), S Shoot Length (SHL), Shoot Fresh Weight (SFW), Shoot Dry Weight (SDW), Electrolyte leakage (EL), Total phenol content (mg GAE/g) (TPC), Total Flavonoids (µg QE/g), Catalase (µ Mol/g/min) (CAT), Ascorbic peroxidase (µ Mol/g/min) (APX).

It's well understood that, Eos yield and composition varies with plant development and environmental factors. Significant changes were observed in the composition of EO s and the yield.

The total number of compounds identified in all the treatments are 22, 13, 24, 9 and 8 in control, and 25, 50, 75 and 100 mM of NaCl treatments respectively, as illustrated in Figure 3. These phytochemicals represented 92 (control), 93.7 (25 mM NaCl), 95.35 (50 mM NaCl), 95.3 (75 mM NaCl), and 91.2 % (100mM NaCl) to 95.35% of the extracts and their quantity differed in different treatments.

Styrene (11-32%), and different alkanes including, dodecane, tetradecane, and hexadecane were the major components of the extracts. Some compounds, including alpha-methylstyrene, decane, spathulenol, were reduced during salinity stress compared to the control. On the other hand, compounds such as styrene, tridecanol, germacrene-D, beta-ionone, beta-bisabolene, and caryophyllene oxide increased under salinity stress compared to the control treatment. Beta-ionone could inhibit MDA-MB 435 cells proliferation by regulating MAPKs pathway. It may be one of the effects of beta-ionone in anticancer beta-Bisabolene: use of β-bisabolene in the treatment of breast cancers (Yeo et al., 2016).

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Caryophyllene oxide: Overall, these findings suggest that CPO can interfere with multiple signaling cascades involved in tumorigenesis and used as a potential therapeutic candidate for both the prevention and treatment of cancer (Park et al., 2011).

An increase of essential oil yield with salinity has been reported previously in other plant species, e.g. sage (*Salvia officinalis* L.) (Hendawy & Khalid, 2005) and peppermint (*Mentha piperita* L.) (Abou El-Fadl, Abd-Ella, & Hussein, 1990). The stimulation of essential oil production under a moderate degree of salinity could be due to a higher oil gland density and an increase in the absolute number of glands produced prior to leaf emergence (Charles et al., 1990). Salt stress may also affect the essential oil accumulation indirectly through its effects on either net assimilation or the partitioning of the assimilate among growth and differentiation processes (Charles et al., 1990). In contrast, Ansari, Frooqi, and Sharma (1998) suggested that the essential oil content and yield decreased with an increase in water salinity in three *Cymbopogon* species (*C. winterianus*, *C. flexuosus* and *C. martinii*).

The exact cause of the increase in some compounds and the decrease in others in the plants tested under salinity is not known. Changing in quantity of various secondary metabolites may be related to up-regulation of salinity stress associated enzymes which are involved in the biosynthetic pathways of these phytochemicals. Similar to the results obtained in the present study, other experiments have reported changes in the type and amounts of secondary metabolites in other plants. In an experiment performed on *Salvia officinalis*, cineol had the highest percentage at 50 and 75 mM salt and manool had the highest percentage at 100 mM (Taarit et al., 2009).

Conclusion

Salinity stress of different sodium chloride concentrations caused considerable alteration in morphological, physiological characteristics and secondary metabolites of *B. nigra in vivo* and *in vitro* culture conditions. The results showed that all growth-related traits and SPAD were decreased both *in vivo* and *in vitro* with the increased salinity concentrations. However, regarding these traits, there was no significant difference between 50 to 100 mM NaCl concentrations. Total phenolics and flavonoids content of plants at 50 mM NaCl, but at higher stress levels (100 mM NaCl), the amounts were decreased significantly. Among the NaCl treatments, the highest DPPH scavenging activities (IC50) under greenhouse and *in vitro* conditions were detected at 50 mM and 25 mM concentrations, respectively. In general, based on the results, with increasing the salinity level to 75 mM, the activities of CAT and APX were significantly upregulated in both greenhouse and *in vitro* culture conditions. With shifting salinity stress, the type and the amount of the identified essential oil compounds changed. Compounds such as styrene, tridecanol, germacrene-D, beta-Ionone, beta-bisabolene, and caryophyllene oxide increased compared to the controlled treatment.

Material and methods

Plant and growth, maintenance and salinity treatment *in vivo*: Seeds of *B. nigra* were soaked in warm water (50 ˚C) for 24 hours, and sown in soil surface of 70-hole planting trays (five seeds in each hole) on the containing cocopeat and perlite in a ratio of 1: 1, while the seed surface with a thin layer of perlite was covered. To prepare the pot cultivation bed, soil samples were air dried in a greenhouse and passed through a sieve for uniformity and mixed with an equal amount of completely rotted animal manure. In each pot with a diameter of 20 cm and a height of 18 cm, 3

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kg of prepared dry soil was poured. Finally, the seedlings were transferred to the prepared pots in a four-leaf stage.

Sodium chloride treatment: Salinity treatments were applied at concentrations of 0, 0.71, 1.81, 2.9, and 4 NaCl (g/kilogram of soil). This is equivalent to electrical conductivity (EC) of 2, 2.28, 4.57, 6.85, / 14. 9 dS/m (1- dSm), respectively. To calculate the amount of salt required for 3 kg of soil in each pot, a given amount of the soil was placed in a container, saturated with sufficient amount of water, and weighed with a precise scale, then put it in an oven at 70 degrees Celsius and after 24 hours, it was weighed again and the following formula was used to obtain the percentage of soil saturation:

Saturated moisture% = (weight of saturated soil-weight of dry soil) / (weight of dry soil) ×100%

The percentage of soil moisture saturation indicates the amount of water added to a dry soil until it reached saturation. Various concentrations of sodium chloride were added according to the maximum desired electrical conductivity, and after 24 hours the electrical conductivity was measured. Salt treatments were applied at four times using field capacity at intervals of 4 days, and the plants were irrigated until the harvest.

Plant harvest and drying

After one month and observing the signs of stress and budding, *B. nigra* plants were harvested and placed in a dark room (for 10 days) separately on spaced papers, so that the plant dries slowly, and the light does not affect the plant's secondary metabolites. Before harvesting the plant, fresh samples were prepared from the leaves and shoots to measure the content of phenolic compounds, flavonoids, chlorophyll, catalase (CAT) and ascorbic peroxidase (APX) enzymes and DPPH.

Plant growth, maintenance and salinity treatment *in vitro*:

The seeds were washed with distilled water for 10 minutes, followed by soaking in 70% alcohol for 10 minutes. Seeds were surface sterilized in 1% sodium hypochlorite solution (20 ml of 5% normal bleach was diluted with 80 ml sterile distilled water), and two drops of dishwashing liquid for 20 minutes, while shaking. The seeds were planted in the jars containing MS basal medium and incubated in the growth chamber.

When the *B. nigra* seedlings had sufficiently grown, their outer surface was sterilized by spraying 70% ethanol to obtain sterile explants. The explants used included 1 cm pieces of stem and stem tip so that each piece had one node and leaf. Explants were placed in MS medium containing 5 µmol benzylaminopurine (BAP), 1 µmol indole acetic acid (IAA), and different NaCl concentrations (0, 25, 50, 75, 100 mM NaCl). After cultivation, the cultures were transferred into a growth chamber with a temperature of 24 °C under a 16-h photoperiod. After 4 weeks, seedling traits were recorded in culture medium.

Measurement of growth and morphological characteristics:

The growth and morphological traits of control and NaCl treated plants were studied including root and shoot length (cm), their fresh and dry weight (g), number of leaves and leaf area (using T Device Δ leaf area meter, UK) were recorded in plants cultured both *in vivo* and *in vitro* conditions. The dry weight was measured after 48 h of frying the plant material in air dry oven at 70C.

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Physiological parameters

Measurement of Electrolyte leakage (%)

To evaluate the cell membrane stability (CMS), 10 ml of double-distilled water was first poured into glass jars. Then, several discs were prepared from two young and developed leaves from the top of the vegetation one week before harvest and placed on jars. After 24 hours in the dark, the electrical conductivity (E0) of each jar was measured by an EC meter. The jars were placed in an autoclave for 20 minutes, and after cooling, their electrical conductivity was measured again as the maximum electrolyte (EC1) output from the leaf. Finally, the following formula was used to calculate the CMS:

$$CMS\% = (EC0/EC1) \times 100$$

Measuring the greenness of the plant

To study the changes of the leaf's greenness in different salinity treatments, the chlorophyll content of the whole leaf of the plant was measured using a manual chlorophyll meter (model MINOLTA-502, made in Japan).

Antioxidant activity assay / Radical scavenging assay:

In brief, 10 µl of the methanol extract of B. nigra were added to 1 ml of 0.004% methanol solution of DPPH. After 30 min the remaining DPPH was determined at 517 nm using a UV–visible single-beam spectrophotometer (UK). DPPH inhibition percentage (I%) was computed using the following formula:

$$I\% = [(A \text{ control} - A \text{ sample})/A \text{ control}] \times 100$$

Where control is the absorbance of control reaction;half-maximal inhibitory concentration (IC50) was considered as the concentration of the extracts showing 50% of inhibition in DPPH, as calculated from the graph, plotting inhibition against different concentrations of extracts.

Measurement of total flavonoids

Total flavonoids were measured based on the formation of flavonoid-aluminum complex at an absorption wavelength of 415 nm (Huang et al 2004). First, one gram of aluminum chloride was weighed to 50 ml with methanol. Concentrations of 10, 20, 40, 80 and 100 µg/ml quercetin were used to draw the standard curve. The reaction mixture consisted of 1.5 ml of methanol, 100 µl of aluminum chloride, 100 µl of sodium acetate, 500 µl of extract and 2.8 ml of distilled water. The control solution contained all of the above compounds except the extract. The samples were then kept at room temperature for 30 minutes and their absorbance was measured at 415 nm. The content of flavonoids was expressed in micrograms of quercetin per gram of extract.

Total Phenol measurement

The amount of phenol was determined using folin reagent. Folin Siocalto 2 solution was diluted with an equal volume of distilled water. 20 g of sodium carbonate was weighed and diluted in 100 ml distilled water. Then 1.5 ml methanol, 2 ml of folate, 500 µl of extract were mixed, and after 5 minutes, 3.75 ml of the prepared sodium carbonate solution was added. The resulting reaction mixture was kept at room temperature for 90 minutes and their absorbance was measured at 725 nm. The control solution contained all of the above ingredients except the extract. The total phenol content of each sample was expressed in terms of Gallic acid equivalent (GAE) according to the standard sample.

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Measurement of catalase (CAT) and ascorbic peroxidase (APX) activity

Preparation of extract for measuring enzyme activity: For preparing 500 ml of extraction buffer, 6.7 g of Tris with 0.5 g of PVP were dissolved in 450 ml of distilled water. Then, using hydrochloric acid (1N), the pH of the solution is brought to 8 and the solution is brought to a final volume of 500 ml. 0.5 g of leaf sample was dissolved in 2 ml of extraction buffer and thoroughly homogenized in a mortar previously cooled in the freezer. The resulting mixture was centrifuged in a tube for 10 minutes at 13,000 rpm, followed by taking the upper phase to read the activity of the enzymes.

The activity of CAT enzyme was measured using 20 µl of enzyme extract mixed with 980 µl of phosphate buffer containing 2 mM hydrogen peroxide. The adsorption changes of the samples over the time (OD/min) were measured using a spectrophotometer at 240 nm. Enzymatic activity was calculated using Beer Lambert's law with a catalase extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ and finally expressed in micromoles per gram of fresh tissue per minute. To measure the APX activity, 50 µl of the extract was mixed with 1 ml of measuring solution containing 50 mM potassium phosphate buffer (pH = 7), 0.1 mM EDTA, 0.5 mM ascorbic acid and 0.15 mM hydrogen peroxide (H_2O_2). Sample absorptions (after 1 minute) was read with a spectrophotometer at 290 nm. One unit of APX is equivalent to the breakdown of one mM ascorbic acid per minute.

Essential oil's extraction and Gas chromatography-mass spectrometry (GC-MS)

The aerial parts of the plant, including the stems and leaves, were dried at room temperature after harvest powdered with an electric grinder and mixed with n-hexane solvent by maceration method for 7 days with occasionally shaking. Finally, the mixture is filtered using filter paper and then centrifuged to discard the solid residues. The extracts were kept in lid closed containers in refrigerator until analysis.

Gas Chromatography: The extracts were analyzed by GC–MS using Agilent 7890A Network GC system combined with Agilent 5975C Network with Triple-Axis Detector. GC analysis was equipped with a splitless model injector (with 1.0 µm volume and 250 °C temperature). HP 5MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm) was used and helium served as the carrier gas (1.1 mL/min). The column pressure was fixed at 8.13 PSI. Oven temperature initially was kept at 50 °C for 2 min after injection and then increased to 250 °C with an 8 °C/min heating ramp and kept constant at 250 °C for 2 min. A hydrocarbon mixture for retention index (RI) measurement was injected under above conditions.

Gas chromatography-Mass Spectrometry (GC-MS): MS was performed with an ionization voltage of 70 eV, and mass range of 34–500 m/z. The 280°C and 250°C were used as anion source and interface temperatures, respectively. The retention indices of the compounds were calculated, based on C8-C20 series of alkane.

Identification of essential oil components (Eos): Constituents of the essential oils (Eos) were identified by matching their retention indices and mass spectra pattern with related available data and/or with the authentic compounds. Additionally, the identification was made by matching with Wiley NBS mass spectral library and literature (NIST Chemistry WebBook) (Adams, 2007; Linstrom and Mallard, 2001). In addition, retention indices of the main constituents were comprised with those of authentic compounds to confirm their precise identification. To quantify the percentage of the individual constituents, the peak area of each constituent was divided by the total peak area of all essential oil compounds.

Statistical analysis

All experiments *in vivo* and in *vitro* were performed in a randomized complete block design with five treatments, three replications. Analysis of variance of was performed using statistical software, Ver 22 SPSS, and the mean comparison was performed using, Duncan's multiple range test at 0.05 significant level. Principle Component Analysis (PCA) was applied to evaluate association between variables measured in greenhouse condition under different NaCl treatments. To carry out PCA procedure, a total of 165 data (55 × 3 repeats) were analyzed, and then the eigenvalues were calculated using a correlation matrix among the variables, and the two-dimensional score and loading plots were created.

Acknowledgements:
NM, MYH, ZD and SO would like to appreciate the University of Tabriz for supporting and funding the research. VT and KR acknowledges the support of FAMU Department of Biological Sciences and FAMU NSF REU for supporting to conduct the research.

Authors' contributions:
Mehdi Younessi-Hamzekhanlu: Methodology, Data curation, Writing original draft; Zahra Dibazarnia: Methodology, Data curation, Software; Shahin Oustan: Conceptualization, Formal analysis, Validation; Vinson Teniyah: Formal analysis, Investigation, Software; Ramesh Katam: Formal analysis, Fund acquisition, Writing editing, reviewing; Nasser Mahna: Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing editing. reviewing.

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