

Article

Mild Salinity Stimulates Biochemical Activities and Metabolites Associated with Anticancer Activities in Black Horehound (*Ballota nigra* L.)

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Abstract: Black horehound (*Ballota nigra* L.) is one of the most important medicinal plants, as a rich source of health-promoting essential oils and metabolites. Salinity stress affects plant development and alters antioxidant activity and plant metabolite composition. The present research aimed to study the effect of salinity on physiological and biochemical changes and 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 morphological characteristics of the plant were measured. The growth-related traits and soil plant analysis development (SPAD) were decreased both in vivo and in vitro. Additionally, increased salt concentration negatively affected the cell membrane integrity. The total phenolic content and flavonoids of plants growing in the greenhouse increased by 21% at 50 mM of NaCl, but the amounts decreased significantly at higher stress levels (100 mM of NaCl). Antioxidant activity was also measured. Among the NaCl treatments, the most increased DPPH scavenging activities (IC₅₀) under greenhouse and in vitro conditions were detected at mild salinity stress, but the activity significantly decreased in higher salinity treatments (i.e., 75 and 100 mM). In general, 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 was obtained. Salinity level caused a shift in the metabolite expression. Mild salinity stress elevated the metabolites involved in anticancer and anti-inflammatory activities, such as β -ionone and caryophyllene oxide. However, the higher salt stress resulted in a significant reduction in their expression. Differential expression of metabolites to various levels of salt stress can be further exploited for the in vitro biosynthesis of metabolites.

Keywords: antioxidants; *Ballota nigra*; essential oils; salinity



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1. Introduction

Soil salinity is an important abiotic stress factor that limits plant growth, affecting agricultural production and crop sustainability. To date, nearly 20% of the world's agricultural land is under saline conditions; thus, its impact on severe losses is estimated to be USD 12 billion each year [1]. Soil salinity has increased over time and is more prevalent in arid and semi-arid regions where frequent drought occurs, perturbing high evaporation in plants [2]. It is predicted that soil salinity will increase exponentially in the wake of climate change [3]. During this process, various salts accumulate in the surface layers of the soil, thus exposing the plants to secondary drought stress [4].

Ballota nigra L. is a perennial medicinal plant belonging to the mint family, Lamiaceae. It is widely distributed in temperate and subtropical regions in Europe and the Middle

East. The plant inhabits waste-filled environments and roadsides in human settlements, as well as weedy places and cultivated grounds [5]. Species of *Ballota* L. are traditionally used for cardiovascular diseases and the inflammation of the respiratory tract. Other medicinal purposes include treating nausea, vomiting, nervous dyspepsia, and producing mildly astringent sedatives, and antimicrobial agents [6]. The majority of the global population relies on herbal medicine supplements [7,8]. However, the quality and quantity of these herbal metabolites totally depend on environmental conditions such as soil salinity and drought.

High salt concentrations in the soil can cause detrimental effects on plant growth and development, germination, yield, nutrient uptake, photosynthesis, quality, and quantity of primary and secondary metabolites. Salt stress affects medicinal plants through different physiological stages, including seed germination, seedling growth by blocking the mobilization of reserve foods, injuring hypocotyls, and suspending cell division [9,10]. A similar impact on morphological characteristics such as leaf number, leaf area, and biomass was observed in several medicinal plants [11,12].

Salinity stress leads to oxidative stress in plants and an increase in reactive oxygen species (ROS). These oxygen-derived radicals reduce target precursor biomolecules essential for metabolic processes and inhibit their synthesis [13]. In addition, ROS attack vital cell macromolecules such as nucleic acids, proteins, and lipids and cause mutations in the structure of DNA, alter the nature of proteins, and damage membranes [14–16]. Proline content increases as a stress osmolyte increases under salinity stress in plants such as peppermint, sage, and summer savory [17]. Salinity stress affects the production of secondary metabolites and essential oils in medicinal plants [18]. The composition of essential oil, phenolic compounds, proline, and photosynthetic pigments is differentially expressed in the plants grown under salinity conditions [19]. The levels of terpenoids such as menthol in mint, carvacrol in coriander, 1,8-cineole in sage, and linalool in basil increased with increasing salinity [20]. In contrast, metabolites such as gamma-terpinene in coriander, p-cymen in marjoram, and eugenol were reduced in basil [21,22].

Due to the significance of fragrant species in terms of medicine, understanding the mechanisms underlying salt tolerance in medicinal plants has gained importance, with relevance to the production of essential oils and other secondary metabolites. Developing salt-tolerant medicinal plants leads to increased raw materials for drugs, flavors, fragrances, and spices. To increase the production of a medicinally essential compound in plants, it is necessary to understand the distribution of the metabolites in the plants in response to various levels of salt stress. Therefore, this study was carried out to determine the effects of sodium chloride on morphological, physiological, and biochemical characteristics, and the metabolites of *B. nigra*.

2. Results and Discussion

2.1. Soil Salinity Inhibited Plant Growth and Yield

Analysis of variance showed that salinity has a significant effect ($p < 0.01$) on all growth-related traits in vivo and in vitro (Table 1). Plant morphological traits such as leaf number, leaf area, root and shoot length, fresh and dry weights of both root and shoot tissues, soil plant analysis development (SPAD), and branch numbers were significantly reduced by NaCl (25 mM to 100 mM)-induced salt treatment (Table 2).

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

S.V	d.f	Mean of Squares (Greenhouse)									Mean of Squares			
		LN	LA	RL	RFW	RDW	SHL	SFW	SDW	SPAD	d.f	LN	SHE	NB
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

LN: leaf number, LA: leaf area, EL: root length, RFW: root fresh weight, RDW: root dry weight, SHL: shoot length, SFW: shoot fresh weight, SDW: shoot dry weight, SPAD: soil plant analysis development, NB: number of branches. ** shows a significant difference at 5% significance level ($p \leq 0.05$).

Table 2. Effect of salinity stress on physiological parameters measured in the greenhouse and in vitro.

		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
In vitro	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 ± 1.5 a	35.3 ± 0.3 b	33.5 ± 0.3 b	29.3 ± 0.2 c	24.7 ± 1.2 d
	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 branches	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% significance level ($p \leq 0.05$).

Leaf area and number decreased after the plant was exposed to a higher concentration of NaCl solution. Plants with reduced leaf area exhibit a low evaporation rate and prevent water loss, resulting in a reduction in photosynthetic machinery [23]. Exposure to higher NaCl levels caused severe damage to photosynthetic tissues and reduced leaf gas exchange due to the ion concentration in leaf tissues [24]. 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 [25,26]. Previous results have shown that the shoot length fresh weight of *B. nigra* decreases significantly due to increasing salt concentration [27].

SPAD value was reduced by increasing the NaCl concentration (Table 2). Reduction in SPAD value following photosynthetic pigments under salt stress leads to a reduction in the catalytic activity of the aminolevulinic acid synthase enzyme. This, in turn, catalyzes the production of 5-aminolevulinic acid, a key precursor in the chlorophyll pigment biosynthesis pathway [28]. A similar reduction in the growth indices of medicinal plants has been earlier reported in basil, *Salvia* spp., *Carthamus*, and *Dracocephalum* [29].

2.2. Effect of Salinity on Cell Membrane Integrity

The cell membrane stability was severed in the plants treated with higher saline conditions. The results show that NaCl concentration at 50 mM and above result in an increase in electrolyte leakage compared to the in vivo grown control plants (Figure 1; Table 3). Due to their high energy and reactivity, these ions damage many cellular compounds such as fats, proteins, carbohydrates, and nucleic acids, and by altering membrane structure, causing electrolyte leakage [30,31]. Higher salt concentrations cause damage to the plasma membrane through the decline of the K^+/Na^+ ratio and the production of reactive oxygen species, such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals [32].

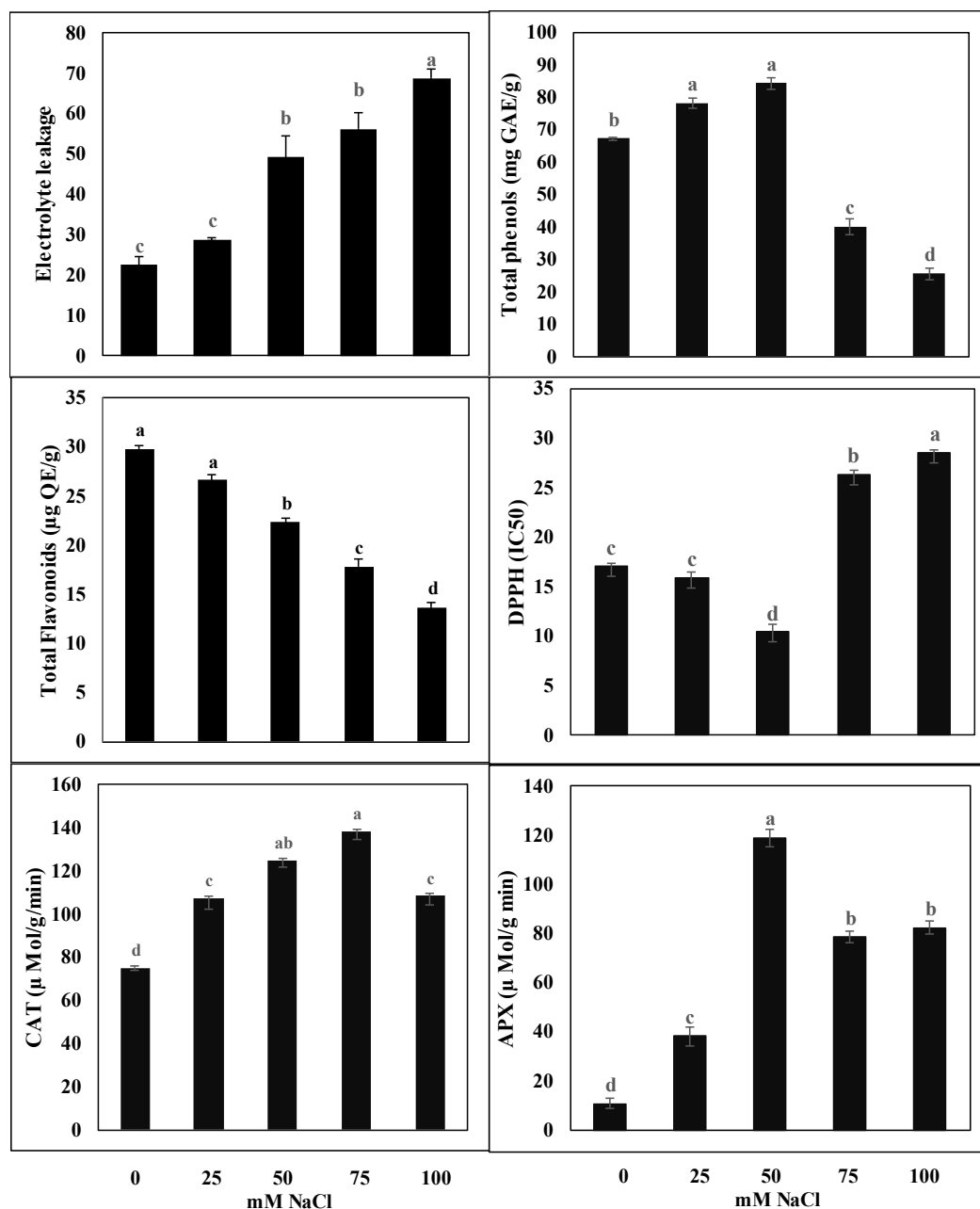


Figure 1. The effect of salinity stress on physiological and biochemical parameters measured in the greenhouse. Means along with the same letter are not significantly different at 5% significance level ($p \leq 0.05$).

Table 3. ANOVA of salinity stress effect on physiological parameters measured in the greenhouse and in vitro.

Mean of Squares								
Greenhouse	Source of variation	d.f	TPh	Flv	DPP	APX	CAT	EL
	Repeat	2	4.5	0.2	0.5	2391.3	0.8	4.63
	Salinity	4	1916.5 **	126.5 **	171.2 **	132,662.0 **	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

Table 3. Cont.

Mean of Squares								
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	-

* and ** show a significant difference at 1% ($p \leq 0.01$) and 5% significance level ($p \leq 0.05$), respectively.

2.3. Effect of Salinity on Total Phenols, Flavonoids, and Antioxidant Enzyme Activities

Plant secondary metabolites such as phenol and total flavonoids have a strong potential for scavenging free radicals. These compounds act as electron donors and may neutralize unwanted reactions caused by free radicals in living organisms [33]. The presence of total phenolic compounds often correlates to the antioxidant activity of plant products. In this study, the total phenol contents were initially found in higher amounts in vitro cultures than in vivo. These results are similar to earlier reports that observed in vitro cultures of *B. nigra* [34]. Total phenol contents increased by 20% in response to moderate salt stress (50 mM of NaCl) and started declining at higher salinity levels (75 and 100 mM of NaCl) in both in vivo and in vitro plants (Figures 1 and 2). Peppermint and rosemary extract has shown high antioxidant activity, which is directly related to the higher phenolic content of the plants [35]. Greenhouse-grown plants showed higher concentrations of total flavonoids compared to that of plants in vitro. However, their values significantly reduced in response to high salinity treatments.

On the contrary, the total flavonoids increased in response to NaCl treatments in vitro. Earlier studies showed a reduction in total phenol and flavonoid contents due to salt stress [36]. In general, the number of flavonoids in in vitro decreases compared to in vivo conditions; however, an acceptable number of these compounds is still produced in in vitro grown plants, suggesting in vitro cultures' potential for secondary metabolite production.

2.4. Antioxidant Activity

DPPH Radical scavenging activity: Free radical scavenging activity (DPPH) at IC50 was measured to assess the antioxidant capacity, representing the amount of *B. nigra* methanolic extract that can remove 50% of the DPPH radical in the environment. Among the NaCl treatments, the highest DPPH scavenging activity (IC50) in in vivo and in vitro conditions was detected in 50 mM and 25 mM concentrations, respectively (Figures 1 and 2). However, under in vitro conditions, treatments with 25 and 50 mM of NaCl did not show a significant difference. The strongest DPPH scavenging activity (IC50) was observed (both in vivo and in vitro grown plants) at concentrations of 25 and 50 mM of NaCl, in which the number of phenolic and flavonoid compounds as antioxidant compounds was at a maximum (Figures 1 and 2).

Catalase and Ascorbate peroxidase enzyme assay: Catalase (CAT) and ascorbate peroxidase (APX) enzymes are the important antioxidants, as they improve plant defense systems to deal with abiotic stresses. These enzymes can directly convert hydrogen peroxide to water and oxygen and completely eliminate the toxicity of this oxygen-free radical [37]. Both APX and CAT are enzymatic components of antioxidant machinery with different affinities for hydrogen peroxide, wherein APX is responsible for fine-tuning reactive oxygen species for signaling and is a more efficient scavenger of H_2O_2 , while CAT removes excess ROS under stress [38].

Our results indicate that CAT levels were higher in vivo than in vitro. The CAT activity increased up to 75 mM in vivo, while their levels increased up to 50 mM in vitro. It should be noted that the increase in vitro was higher (65%) than in vivo (45%) with respect to the control. APX was found in similar levels both in vivo and in vitro, and their levels were elevated to moderate levels of salinity stress (50 and 75 mM of NaCl, respectively) under both growth environments. NaCl at 100 mM was detrimental for both the enzymes, as the activity of both enzymes reduced significantly at this higher salt stress.

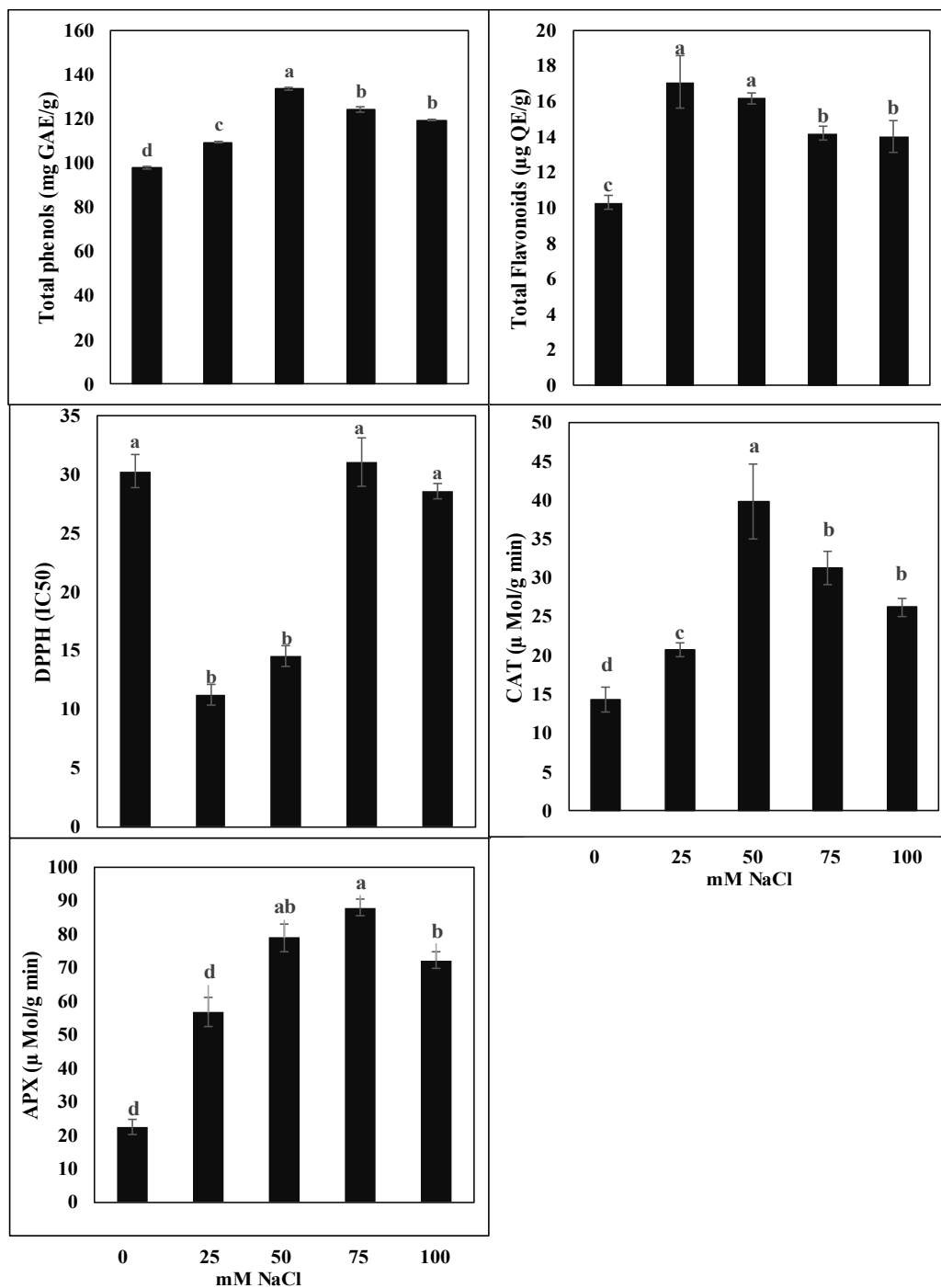


Figure 2. The effect of salinity stress on physiological and biochemical parameters measured in vitro. Means along with the same letter are not significantly different at 5% significance level ($p \leq 0.05$).

In this experiment, the high amounts of APX in both in vivo and in vitro cultures suggest its role in regulating ROS for signaling under high-salt stress conditions (NaCl > 75 mM). APX is widely spread in cell organelles and detoxifies H_2O_2 in response to environmental stresses [39]. Several transcription factors are reported to regulate APX genes and improve plant tolerance against waterlogging stress [40]. APX is shown to increase in salt-tolerant cultivars, while it significantly diminishes in salt-susceptible cultivars of potato [41]. The observed enzyme (CAT and APX) values in response to NaCl stress in our studies were higher than some of the corresponding published data obtained in salt-tolerant cultivars when compared to susceptible potatoes. The decrease in the activity level of these

enzymes at a 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 the *B. nigra* plant. In a 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 [42].

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 in response salt stress are still not clearly understood. 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+}), phosphorylation, redox-dependent post-translational modifications, and transcript factors [43].

ANOVA results show that total phenol and flavonoid content, DPPH (IC50) activity, APX, and CAT activity are significantly affected ($p < 0.01$) by NaCl stress in vivo and in vitro (Table 3). The first two principal components, PC1, and PC2, explain 83.6 and 12.3% of the whole variance, respectively (Table 4). According to the eigenvectors of all variables in every two principal components, the correlation between the variables can be deduced from the biplot (Figure 3). The correlation of CAT and APX enzymes was positive but negative for total phenol content and DDPH (IC50), respectively. Based on the direction of the variables, vectors, and treatment points, it is inferred that the activity of two enzymes and the total phenol content (TPC) reached their maximum value at 50 mM of NaCl.

On the other hand, DPPH scavenging activity (IC50) and membrane integrity (electrolyte leakage) significantly decrease in higher salinity treatments (i.e., 75 and 100 mM). Overall, our findings corroborate the results obtained by mean comparison analysis. The minor change observed is only due to two components, which, while unable to entirely explain the variability, did explain a large portion of it (95.9% of the whole variation).

Table 4. PCA is based on the variables measured in greenhouse 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 ($\mu\text{Mol/g/min}$)	−0.21	0.36
APX ($\mu\text{Mol/g/min}$)	−0.21	0.45
Eigenvalues	12.5	1.8
% of variance	83.6	12.3
Cumulative %	83.6	95.9

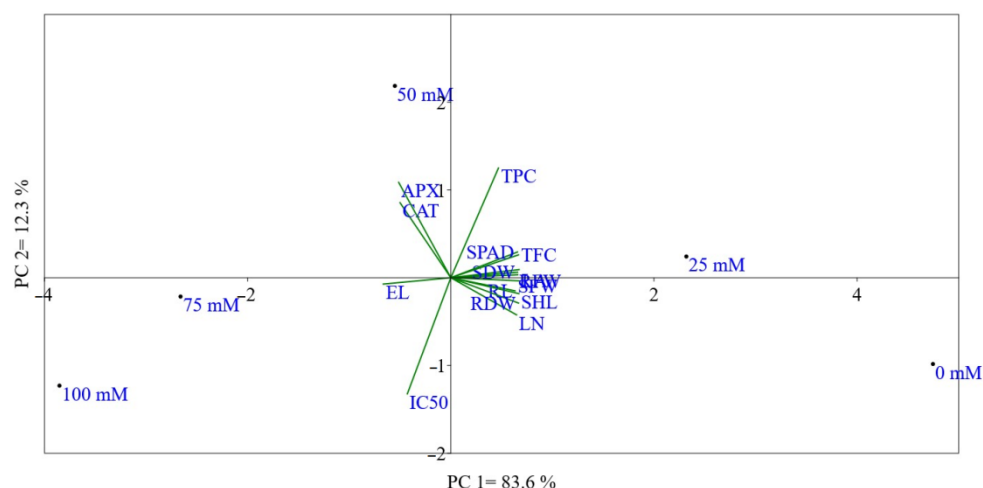


Figure 3. Biplot resulting from PCA procedure for all the variables measured in greenhouse conditions under different NaCl treatments (0, 25, 50, 75, and 100 mM). Leaf number (LA), leaf area (LA), root length (RL), root fresh weight (RFW), root dry weight (RDW), shoot length (SHL), shoot fresh weight (SFW), shoot dry weight (SDW), electrolyte leakage (EL), total phenol content (mg GAE/g) (TPC), total flavonoids (μg QE/g), catalase ($\mu\text{Mol/g/min}$) (CAT), ascorbic peroxidase ($\mu\text{Mol/g/min}$) (APX).

2.5. Salinity Effect on Metabolite Synthesis

Thirty-nine metabolites, including essential oils, are differentially expressed in response to NaCl stress. The GC/MS analysis of hexane extracts of plants grown *in vivo* revealed significant quantitative changes in the metabolite composition in response to NaCl treatments (Figure 4). The total number of compounds identified in all the treatments was 22, 13, 24, 9, and 8 in the control for treatments of 25, 50, 75, and 100 mM of NaCl, respectively. These phytochemicals represented 92 (control), 93.7 (25 mM of NaCl), 95.35 (50 mM of NaCl), 95.3 (75 mM of NaCl), and 91.2% (100 mM of NaCl) to 95.35% of the extracts, and their quantity differed in different treatments.

Among the total metabolites identified in the extract, styrene (11–32%) and alkanes including decane, dodecane, and tetradecane were detected in more abundant quantities. These compounds have shown carcinogenic activity and are asphyxiants and skin irritants, and thus have adverse health effects [44–46]. The data show that mild salinity stress (25–75 mM) elevated the composition of essential oils benzimidazole, β -bisabolene, β -ionone, caryophyllene oxide, hexadecane, methyl oxyphenyl, naphthalene decahydridimethyl, phytol, propene-1-one, quatephenyl, sesquiphallendren, and triphenyl benzene following a decline at severe stress, indicating the diversity of metabolite responses to various stress levels. An increase in essential oil yield with salinity has been reported previously in sage [47]. Several of the metabolites that increased due to mild salt stress reportedly function as anticancer agents through various molecular mechanisms.

β -ionone inhibits MDA-MB 435 cells' proliferation by regulating the MAPKs pathway. β -bisabolene is most commonly used in the treatment of breast cancers [48]. Caryophyllene oxide interferes with multiple signaling cascades involved in tumorigenesis and is used to prevent and treat cancer [49]. A terpenoid, sesquiphellandrene, and heterocyclic organic compound, benzimidazole, demonstrated cytotoxic activity against various cancer cell lines [50]. These derivatives of 1,3-diphenyl-2-propen-1-one, which is a flavonoid precursor, are known to have genotoxic, cytotoxic, antigenotoxic, and anticytotoxic activities [51]. Phytol, a chlorophyll component, is an antiarthritic agent that interacts with key inflammatory pathways of arthritis. Methyloxyphenyl is used as a muscle relaxant, while phytol attenuates inflammatory reactions, decreasing the release of cytokines [52,53]. Naphthalene deca hydro dimethyl is a volatile compound of essential oils most commonly present in flowers exhibiting antifungal properties [54].

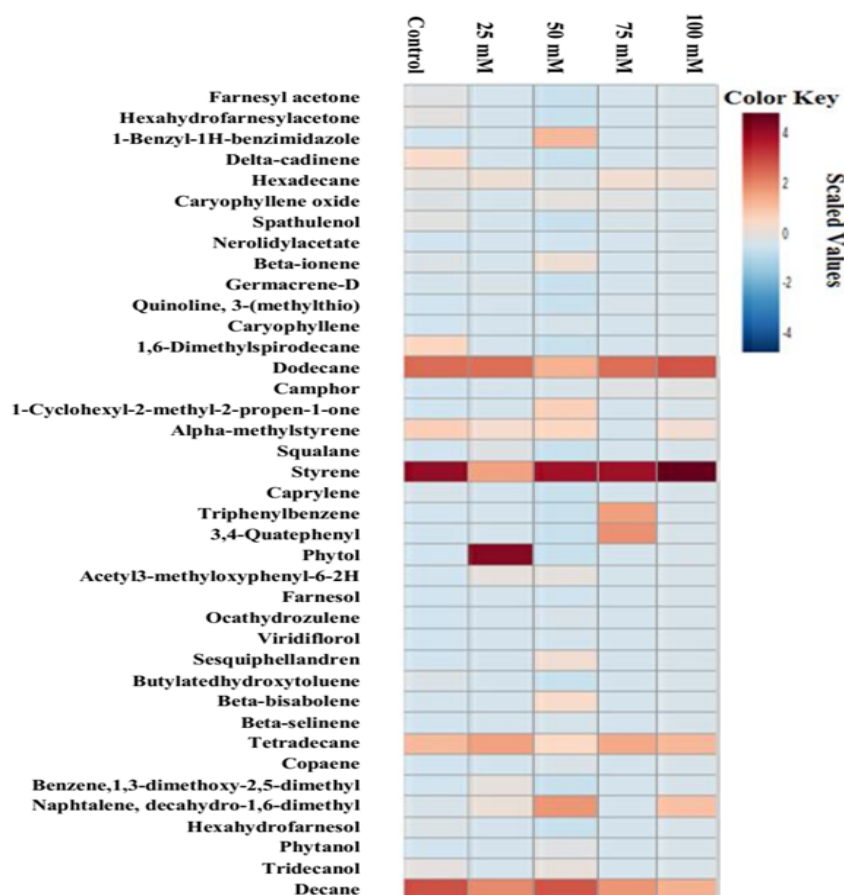


Figure 4. The heat map of detected compounds under different NaCl concentrations.

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. An excess of sodium (Na) in the cytoplasm inhibits several enzyme activities. It also disrupts potassium absorption, causing a high Na/K ratio, reducing plant growth, and increasing oil gland density before leaf emergence [55]. In an experiment performed on *Salvia officinalis*, cineol had the highest percentage at 50 and 75 mM of salt, and manool had the highest rate at 100 mM [56].

It is noteworthy that in the present studies and published literature, the metabolites of therapeutic value have shown an increase in response to mild salt stress up to 75 mM of NaCl. Furthermore, we observed an increase in the metabolites, triphenyl benzene, quatephenyl, and hexadecane, which are volatile phytotoxic compounds and were found to be soil contaminants [57]. Known antibacterial, antifungal, anti-inflammatory compounds delta cadenine, tridecanol, 1,6, dimethylspirodecane, and alpha-methyl styrene were reduced during salinity stress [58–61]. Salinity levels decreased the essential oil (EO) yield of *Rosmarinus officinalis* L., where 1,8-cineole content decreases up to 50% with increasing NaCl concentrations (from 25 to 200 mM) [62].

3. Conclusions

Salinity stress causes considerable alteration in morphological and physiological characteristics and reduces the cell membrane stability of *Ballota nigra*. All growth-related traits and SPAD were decreased in vivo and in vitro with increased salinity stress at 50 and 100 mM of NaCl. Total phenolics and flavonoids were reduced in response to salinity stress, while an increase in total flavonoids was observed in vitro cultures in response to NaCl. The increased activity of CAT and APX enzymes in response to moderate and high levels of NaCl both in vivo and in vitro suggests that the plant is moderately tolerant to

salt stress. More detailed studies using different cultivars of *B. nigra* provide additional information on the diversity for salt tolerance available in this important medicinal plant.

The level of salinity stress exhibited a shift and a mixed response in the metabolite composition of the plant extract. An increase in the NaCl concentration caused a reduction in the total metabolites. Metabolites, styrene, and certain alkanes, which are carcinogenic, were detected in greater abundance and unaffected by salt stress. The expression of delta cadenine, tridecanol, and similar metabolites known for antibacterial properties was observed, and their anti-inflammatory properties were reduced in response to NaCl treatment. Mild salinity stress elevated metabolites such as β -ionone and caryophyllene oxide with anticancer activities and other properties. However, at higher stress (>75 mM of NaCl), their quantities reduced significantly. Furthermore, the increased levels of these enzymes in cell cultures treated with higher NaCl concentrations plausibly manipulate cellular biosynthesis for flavonoids in vitro to produce secondary metabolites at moderate to high NaCl concentrations.

4. Material and Methods

4.1. Plant and Growth, Maintenance, and Salinity Treatment In Vivo

Seeds of *B. nigra* were soaked in warm water for 30 min [63] and sown in soil surface of 70-hole planting trays (five seeds in each hole) containing cocopeat and perlite at 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, 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 kg of prepared dry soil was poured. Finally, the seedlings were transferred to the prepared pots in a four-leaf stage.

Salinity treatments: Sodium chloride treatments were applied at concentrations of 0, 0.71, 1.81, 2.9, and 4 NaCl (g/kg of soil). This is equivalent to electrical conductivity (EC) of 2, 2.28, 4.57, 6.85, 14.9 dS/m, respectively. To calculate the amount of salt required for 3 kg of soil in each pot, a given amount of soil was placed in a container, saturated with a sufficient amount of water, and weighed with a precise scale, then placed in an oven at 70 degrees Celsius, and after 24 h, it was weighed again, and the following formula was used to obtain the percentage of soil saturation:

$$\text{Saturated moisture\%} = (\text{weight of saturated soil} - \text{weight of dry soil}) / (\text{weight of dry soil}) \times 100\% \quad (1)$$

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 h, the electrical conductivity was measured. Sodium chloride was applied four times using field capacity at intervals of 4 days, and the plants were irrigated until the harvest.

4.2. Plant Harvest and Sampling

The fresh samples were prepared from the leaves and shoots to measure the content of phenolic compounds, flavonoids, chlorophyll, free radical scavenging activity (1,1-Diphenyl-2-picrylhydrazyl-DPPH), catalase (CAT), and ascorbic peroxidase (APX) enzymes. The plant samples were harvested and dried in the dark and stored for further analysis of secondary metabolites.

4.3. Plant Growth, Maintenance, and Salinity Treatment In Vitro

The seeds were washed in distilled water for 10 min, followed by soaking in 70% alcohol for 10 min. Seeds were surface sterilized in 1% sodium hypochlorite solution (20 mL of 5% standard bleach was diluted with 80 mL of sterile distilled water) and two drops of dishwashing liquid for 20 min 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 a 1-cm long stem and stem tip so that each piece had one node and leaf. Explants were placed in

MS medium containing 5 μmol of benzylaminopurine (BAP), 1 μmol of indole acetic acid (IAA), and different NaCl concentrations (0, 25, 50, 75, 100 mM of NaCl). After cultivation, the cultures were transferred into a growth chamber with a temperature of 24 °C under a 16 h photoperiod. After four weeks, seedling traits were recorded in a culture medium.

4.4. 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), and the number of leaves and leaf area (using T Device Δ leaf area meter, UK) were recorded in plants cultured in vivo and in vitro. The dry weight was measured after 48 h of drying the plant material in the air-dry oven at 70 °C.

4.5. Measurement of Electrolyte Leakage (%)

To evaluate the cell membrane stability (CMS) of plants growing in a greenhouse, 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 in the pot. After 24 h in the dark, each pot's electrical conductivity (E0) was measured by an EC meter. The pots were autoclaved for 20 min, and after cooling, their electrical conductivity was measured as the maximum electrolyte (EC1) output from the leaf. Finally, the following formula was used to calculate the CMS:

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

4.6. Antioxidant Activity Assay/Radical Scavenging Assay

In brief, 10 μL of the methanol extract of *B. nigra* was 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 spectrophotometer. DPPH inhibition percentage (I%) was calculated as follows below:

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

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

4.7. Measurement of Total Flavonoids

Total amount of flavonoids was measured based on the formation of the flavonoid–aluminum complex [64]. The reaction mixture consisted of 1.5 mL of methanol, 100 μL of 2% methanolic aluminum chloride, 100 μL of sodium acetate (50 g/L), 500 μL of extract and incubation at room temperature for 30 min. The absorbance was measured at 415 nm, and the flavonoids were expressed in μg quercetin/g extract.

4.8. Total Phenol Measurement

The amount of phenol was determined using the Folin reagent [47]. A total of 20 g of sodium carbonate was weighed and diluted in 100 mL of distilled water. The reaction mixture contained 1.5 mL of methanol, 2 mL of Folin–Ciocalteu reagent, and 500 μL of extract. After 5 min, 3.75 mL of the prepared sodium carbonate solution was added. The resulting reaction mixture was incubated at room temperature for 90 min, and its absorbance was measured at 725 nm. The total phenol content of each sample was expressed in terms of Gallic acid equivalent (GAE) according to the standard.

4.9. Measurement of Catalase (CAT) and Ascorbic Peroxidase (APX) Activity

Five hundred mg of leaf sample was dissolved in 2 mL of extraction buffer (50 mM Tris HCl) and thoroughly homogenized in a previously cooled mortar. The resulting mixture

was centrifuged in a tube for 10 min at 13,000 pm, followed by taking the upper phase to read the activity of the enzymes.

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

4.10. Identification and Characterization of Metabolites

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

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 $^\circ\text{C}$ temperature). HP 5MS capillary column (30 m $\times$ 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 was initially kept at 50 $^\circ\text{C}$ for 2 min after injection and then increased to 250 $^\circ\text{C}$ with an 8 $^\circ\text{C}/\text{min}$ heating ramp and kept constant at 250 $^\circ\text{C}$ for 2 min. A hydrocarbon mixture for retention index (RI) measurement was injected under the above conditions.

MS was performed with an ionization voltage of 70 eV and a mass range of 34–500 m/z . Temperatures of 280 $^\circ\text{C}$ and 250 $^\circ\text{C}$ were used as anion source and interface temperatures, respectively. The retention indices of the compounds were calculated based on the 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 comparing Wiley NBS mass spectral library and literature (NIST Chemistry WebBook) [65,66]. In addition, retention indices of the main constituents were composed of authentic compounds to confirm their precise identification. To quantify the individual components' percentage, each element's peak area was divided by the total peak area of all essential oil compounds.

4.11. Statistical Analysis

All in vivo and in vitro experiments were performed in a randomized complete block design with five treatments and three replications. Analysis of variance was performed using statistical software, Ver 22 SPSS, and the mean comparison was performed using Duncan's multiple range test at 0.05 significance level. Principle component analysis (PCA) was applied to evaluate the association between variables measured in greenhouse conditions under different NaCl treatments. To carry out the PCA procedure, 165 data (55 $\times$ 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.

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