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Original Research

How Salt Stress Represses the Biosynthesis of Marrubiin and Disturbs the Antioxidant Activity of *Marrubium Vulgare* L

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Abstract

We investigated the effects of different concentrations of NaCl (25, 50, 100, and 150 mM NaCl) on growth, phenolic content, and antioxidant activities of horehound (*Marrubium vulgare* L.). The long-term salt treatment (up to 100 mM NaCl) resulted in significant ($p<0.05$) reduction of height, fresh weight, total chlorophyll, and total phenol contents. The negative effect of NaCl was accompanied by a significant restriction in K^+, Ca^{2+}, Fe^{2+}, and Zn^{2+} ion uptake, and by an increase in Na^+ ion concentrations – the effects of which were most pronounced at the highest NaCl level. The content of the main bioactive compound marrubiin decreased with increased NaCl concentrations. The antioxidant activity of the methanol extracts from untreated and salt-treated plants revealed that the extracts from a salt-treated plant with 100 mM exhibits the strongest activity in the DPPH and β-carotene bleaching assays, while it showed no reducing power. The present results suggest that salt treatment negatively affects the morphological, physiological, and biochemical traits of *M. vulgare*, which appears to be highly sensitive to salinity.

Keywords: bioactivity, growth, horehound, phenolic content, salinity

Introduction

Salinity is one of the environmental factors limiting growth, development, and productivity. The deleterious effects of salinity on plant growth are associated with low osmotic potential of soil solution, nutritional imbalance, specific ion toxicity, or a combination of these factors [1]. In addition, salinity induces the generation of reactive oxygen species (ROS) such as the superoxide anions (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical ($^*OH$), and singlet oxygen ($^1O_2$), which can seriously disrupt normal metabolism through oxidative damage to lipids,

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proteins, and nucleic acids [2]. All of these cause adverse pleiotropic effects on plant growth and development at the physiological, biochemical, and molecular levels [3]. The main hallmarks of salt stress are impaired photosynthesis, reduced protein and lipid biosynthesis, and disturbed primary and secondary metabolisms [1].

To cope with salt stress, plants have evolved numerous mechanisms including, among others, the accumulation of compatible solutes (soluble carbohydrates, glycine betaine, polyols, and proline), the induction of enzymatic antioxidants (superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, and glutathione reductase), and the stimulation of non-enzymatic antioxidants (α-tocopherol, β-carotene, ascorbate, and glutathione) [2, 4]. Plant responses to salinity have been extensively studied, and in most cases they were focused on general responses such as growth, photosynthesis, plant nutrition, and some biochemical and molecular parameters [3, 5]. However, little attention has been paid to the effects of salinity on some non-enzymatic antioxidants, namely polyphenols that can play a pivotal role in the mechanism of tolerance/sensitivity of some plant species (particularly medicinal plant species) due to their well-recognized antioxidant activity [6]. The accumulation of phenolics in plant tissues is a distinctive characteristic of plant stress and their induction under different abiotic stress is well documented [7]. This trend is of particular interest due to intriguing biological of phenolic compounds, and any approach leading to overproduction of these valuable compounds – especially in some interesting medicinal plant species – is highly desirable.

Among these medicinal species, horehound (*Marrubium vulgare* L.) has attracted economic interest, particularly in agriculture. In Spain, it has been popularly used on chicken farms to avoid lice, frequent scratching of animals against it to try worming was observed, which intensified planting on farms [8]. In addition, *M.vulgare* also provides in important source for the food and pharmaceutical industries, but only in India, where there are 33 registered medicinal formulations containing white horehound [9]. In 2015, *M. vulgare* preparations were the best-selling herbal dietary supplements, reaching approximately $106 million in retail sales [10]. Certainly, *M. vulgare* is one of the highly demanded Mediterranean species for such multiple biological functions as antioxidant, antimicrobial, anti-inflammatory, cholinesterase inhibitory, antigenotoxic, analgesic, anti-diabetic, anti-oedematogenic, anti-hypertensive, hypolipidemic, antispasmodic, hepatoprotective, and vasorelaxant [9, 11-13, 15]. Most of these properties are attributed to their particular phenolic profile and to the furanoid labdane diterpene lactone marrubiin [16]. However, as for other secondary metabolites, the contents of these valuable compounds are likely influenced by abiotic stress [6]. Indeed, salinity can play the role of elicitor for biomolecular production, including polyphenols. Thus, one may hypothesize that optimal polyphenol yield would be obtained using stress-tolerant species [14]. With regard to this topic, on the basis of increasing soil salinity in Tunisia and its effect on the survival and productivity of many aromatic crops, a major interest is given in the present contribution to understand the effects of salt stress on growth, marrubiin production, phenolic content and the antioxidant activity of an important industrial and economic plant such as *M.vulgare*.

Materials and Methods

Equipment and Reagents

Ion analyses were acquired by atomic absorption spectrophotometer with an Analyst 300 (Perkin Elmer, Norwalk, USA).

UV-Vis spectra were acquired using a Shimadzu 1603 double-beam spectrophotometer. FTIR analyses were obtained in a Nicolet 6700 spectrometer (Thermo, Italy) using a KBr disc. 1HNMR spectra were acquired in a Bruker Avance 400 apparatus at 400 MHZ, using chloroform-d as the solvent. All chemicals and reagents were purchased from Sigma-Aldrich and were of analytical grade.

Plant Material and Salt Stress Treatment

In Tunisia, two varieties of *M.vulgare* (*Typicum* Fiori and *Lanatum* Benth) can be found in all areas except in the extreme south [17]. We chose for study *M.vulgare* var. *Typicum* because it is the most common. Seeds of *M. vulgare* were collected from the region of Boussalem in northwestern Tunisia (latitude 36°36'40.03"N, longitude 8°58'11.36"E, altitude: 141 m) in August 2013. This location was characterized by a low annual rainfall of 700 mm and a mean annual temperature of 16.8°C. Botanical identification was made by Dr. Mouhiba Ben Nasri-Ayachi, a member of the Botanical Laboratory, Faculty of Science of Tunis, according to the Tunisian flora [17]. The seeds were surface-sterilized in 0.5% sodium hypochlorite for 10 min, washed thoroughly with distilled water, and then sown manually in 5 L plastic pots, filled with agricultural soil that had an argyle texture, and maintained under greenhouse conditions. During the pre-treatment, plants were irrigated with distilled water. Forty days after planting, salt treatment was started after obtaining a uniform emergence and establishment of seedlings. Plants were allocated to NaCl treatment using half-strength Hoagland solution supplied with 0, 25, 50, 100, and 150 mM NaCl, renewed every three days for five months. Irrigation continued until harvest except for those treated with 150 mM, which were completely damaged.

Growth and its Component Parameters

For each treatment, measurements of plant height and fresh matter weights were evaluated by destructive harvests of three selected plants from each pot. Plants were harvested at the soil surface and immediately weighed (fresh weight, FW).

Determination of Photosynthetic Pigments

Total chlorophyll contents in the leaves were determined according to the method of [18]. Fresh tissue from the intervenal leaf area was triturated using mortar-pestle with 80% acetone. The photosynthetic pigments were extracted at 4ºC for 24 h in the dark. To measure the content of chlorophyll a, chlorophyll b, and total chlorophyll, the absorbance of samples was recorded at 662 and 645 nm. The content of photosynthetic pigments was expressed as mg· g^{-1} FW.

Determination of Ion Contents

Ion contents were determined in dried *M. vulgare* samples [19] and essayed by an atomic absorption spectrophotometer with an Analyst 300 (Perkin Elmer, Norwalk, USA).

Extraction, Clean-up, and Fractionation

To evaluate the antioxidant activity, fresh vegetable material was first extracted with methanol. After removal of the solvent, the residue was washed with petroleum ether to remove pigments and lipids. The resulting residue was re-suspended in methanol, after which it was filtered through a Whatman No. 4 filter paper, concentrated under vacuum, evaporated to dryness, and weighted (methanol extract).

To evaluate the marrubiin content, the plant was first dried to obtain 6 g of dried material that was extracted with 3 x 100 mL acetone. The acetone extracts were combined and evaporated to dryness and extracted with petroleum ether (15 mL) to obtain the petroleum ether extract. The resulting weight of the acetone extracts were 14.66, 18.92, 37.44, and 19.23 mg of extract/dry material for the control, 25 mM, 50 mM, and 100 mM of NaCl, respectively.

Isolation and Quantification of Marrubiin

For marrubiin extraction, 50 g of dried plant was extracted by percolation with acetone to obtain 18 fractions. Fractions were collected and analyzed by TLC in silica gel plates using cyclohexane-ethyl acetate (6:4). Fractions with the same chromatographic profile were combined to afford five fractions. Fraction 2 (1.01 g) was washed with petroleum ether to remove oil and coloring matter to obtain a residue (0.707 g) that was further fractionated by flash chromatography using an increasing proportion of ethyl acetate in cyclohexane. Fractions containing marrubiin were pooled and crystallized from methanol to afford 131 mg of this diterpene. The identity of marrubiin was confirmed by FTIR and 1H and 13C NMR. Spectroscopic data was identical to the literature [20]. Quantification was carried out based on a calibration curve performed with known amounts of marrubiin by H1NMR as described by [21]. The intensity of the peak

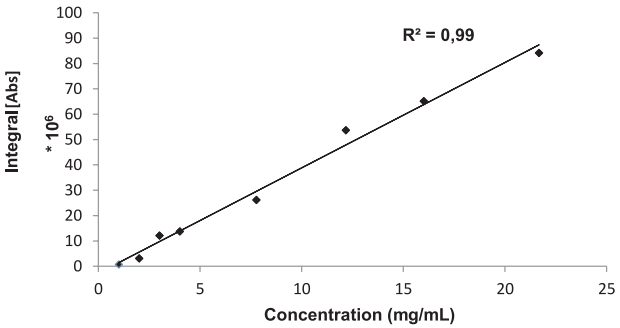

Fig. 1. Calibration curve of marrubiin using the intensity of H-16 at 7.38 ppm.

at 7.38 ppm was used to obtain a calibration curve (Fig. 1):

$$\text{Integral [Abs]} = 4 \cdot 10^6 \text{ [concentration]} - 3 \cdot 10^6 \ (R^2 = 0.99).$$

To quantify the amount of marrubiin, 10 mg of each acetone extract was dissolved in 1 mL of deuterated chloroform and spectra were recorded in the same conditions.

Determination of Total Phenolic Content

The total phenolic compound content in the methanol extract was determined by colorimetric assay using the Folin-Ciocalteu reagent [22] and gallic acid as a standard. Briefly, 1 mL of the methanol solution containing 1 mg of extract was mixed with 4.5 mL of distilled water. One milliliter of Folin-Ciocalteu reagent was added and the contents of the flask were mixed thoroughly. After 3 min, 3 mL of a 2% solution of sodium carbonate was added and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance (Abs) of the mixture was measured at 760 nm. The concentration of total phenolic compounds in methanol extract was determined as milligram gallic acid equivalents per gram of extract (mg GAE/gFW).

The Fe (III) to Fe (II) Reduction

The iron (III) reductive capacity of extract, or ascorbic acid, was evaluated as described by [23]. In brief, 1 mL of dissolved extract with different concentrations was mixed with 2.5 mL phosphate buffer (0.2 M, pH = 6.6), and 2.5 mL of a 10 g·L^{-1} potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution. After 30 min at 50º, 2.5 mL of a 100 g·L-1 aqueous trichloroacetic acid (TCA) solution was added and the mixture was stirred with a glass rod. Finally, a 2.5 mL aliquot was mixed with 2.5 mL ultrapure water and 0.5 mL of a 1 g·L^{-1} $FeCl_3$ solution and the absorbance of the mixtures (Abs) were recorded at 700 nm. A standard graph using ascorbic acid was obtained and the ability of the extracts to inhibit the reduction of Fe(III) is expressed as mg ascorbic acid equivalents per g sample (mg AscAE/g sample).

Radical Scavenging Assay with DPPH Radical (DPPH Assay)

The procedure used in the present study was described before [24]. It is based on the reduction of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical in alcoholic solution, in the presence of a hydrogen-donating antioxidant, due to the formation of a non-radical form, DPPH-H. The absorbance decrease was followed spectrophotometrically at 517 nm and a steady state was reached after 30 minutes. Briefly, an aliquot (25 µL) of the extract solution was placed in a stoppered small flask and 2.5 mL of freshly prepared 0.04% methanolic solution of DPPH radical was added. The mixture was shaken and maintained at room temperature, in the dark, for 30 min. Controls were prepared at the same temperature and with the same solvent used to prepare the extract solution. The radical scavenging activities of the test samples were expressed as the percentage inhibition of the DPPH radical and calculated according to the following formula:

$$E(\%) = [(Acontrol - Asample) / Acontrol] * 100$$

Acontrol and Asample are the absorbance values of the control and the test samples, respectively. Extract concentration providing 50% inhibition (EC50) was calculated using regression analysis in MS excel. The assay was performed in triplicate for each extract.

β-Carotene/Linoleic Acid Assay

The ability of the extract to inhibit the bleaching of the β-carotene-linoleic acid emulsion was determined using the method described by [22] with a slight modification. A stock solution was prepared dissolving β-carotene (20 mg) in chloroform (10 mL). 1 mL of this solution was mixed with 25 µL of linoleic acid and 200 mg of tween 40 into a round-bottomed flask. After removal of chloroform on a rotary evaporator, 50 mL of oxygenated distilled water was added to the flask with vigorous stirring. Emulsion aliquots (2.5 mL) were transferred to a series of tubes containing 300 µL of methanol extract at a final concentration of 1.6 mg·mL^{-1} and were further shaken. The mixtures were immediately transferred to the UV cells and placed in a thermostatized UV-Vis unit at 50ºC and inserted into a Shimadzu 1603 apparatus. The reaction was followed taking readings at regular intervals (120 s) for a period of 2 h. Two blanks, one containing only water and the other containing the same volume of methanol instead of the extracts, were also prepared. The antioxidant activity (AA) of the extracts was calculated using the following equation:

$$AA(\%) = [1 - (A(t = 0) - A(t = 120)/ A0(t = 0) - A0(t = 120)]*100$$

…where AA is the antioxidant activity, A(t = 0) is the absorbance of the solution in the investigation at 0 min, A(t = 120) is the absorbance of the same solution at t = 120 min, and A0(t = 0) and A0(t = 120) are the absorbance of the positive control (methanol without extract) sample at t = 0 min and t = 120 min, respectively.

1H NMR Estimation of Salt Effect on Fatty Acid Content

The extract of petroleum ether using NMR spectroscopy was qualitatively investigated to evaluate the salt effect on the fatty acid content and composition according to [25].

Statistical Analysis

Results were examined statistically using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests, with different levels of salt treatment as factor, and their interactions were performed for the whole data set using "Statistica v.5.1" software [26]. All determinations were conducted in triplicate. Data are expressed as mean ± SD. The differences between individual means were considered to be significant at $p<0.05$.

Results and Discussion

Visual Symptoms

With increasing NaCl concentrations, plant vigor was progressively reduced. Various visual symptoms showing toxicity such as yellowing, necrosis, and stunted growth were observed in NaCl-treated plants (Fig. 2). These symptoms were more evident in the leaves of plants treated with 50 and 100 mM NaCl with a browning appearance compared to control. Plants treated with 150 mM NaCl showed signs of severe damages (chlorotic, necrotic, and wilted leaves), as a result of which all parameters (growth, marrubiin content, phenol, and flavonoid contents as well as their antioxidant activities) measurements could not be performed. These observations were consistent with those observed for *Salvia officinalis* [30], *Salvia scalrea* [31], and *Carthamus tinctorius* [32], among others.

Growth and Chlorophyll Content

As shown in Table 1, a dose-dependent inhibition of growth (estimated in term of fresh weight FW and height) was noticed in NaCl-treated plants. The inhibition of normal growth of plants was possibly due to alteration in cell division, photosynthesis, leaf area expansion, respiration, primary metabolism, ion imbalance or disturbances in ion homeostasis [1, 33]. Literature data about the impact of salt stress on the growth of *M. vulgare* are lacking. According to these authors, the reduction in growth may be an adaptive response to stress, suggesteding that the decrease in growth rate under unfavorable conditions allows for the conservation of energy, thereby helping launch appropriate defensive responses and reducing the risk of damage. Given the significant ($p<0.05$) reduction

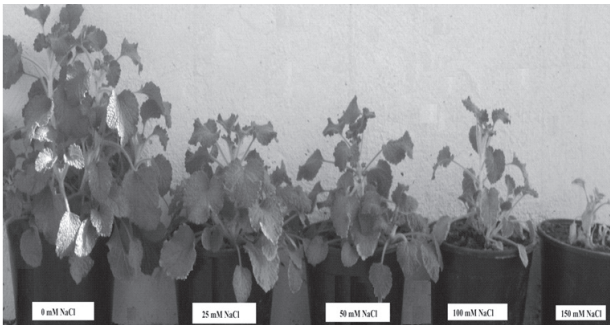

Fig. 2. Effect of salt stress on the growth of *M. vulgare* subjected to different concentrations of NaCl (0, 25, 50, 100, and 150 mM) after five months of treatment.

in FW and height (even at the lowest NaCl concentration of 25 mM), it might be concluded that *M. vulgare* is a salt-susceptible species.

Leaf chlorophyll content (chlorophyll a, chlorophyll b, and chlorophyll a+b) is considered an indicator of general plant health. Salt stress-reduced Chl a and b, resulting in a decrease in total Chl contents in plants treated with different concentrations of NaCl (25, 50, and 100 mM, respectively) (Table 1). This behavior has already been observed in *Aeceras corniculatum*, and the authors have hypothesized that this decrease might possibly be due to the changes in the lipid protein ratio of pigment-protein complexes or increased chlorophyllase activity [29]. The decline in chlorophyll content is believed to be the result of its degradation or inhibition of its biosynthesis under saline conditions [34]. Furthermore, the reduction in chlorophyll content could be due to peroxidation processes in the chloroplast membrane lipids by the reactive oxygen species (ROS) as indicated in previous studies [35]. At this point, it can be speculated that the observed visual symptoms and the reduced growth of *M. vulgare* under saline conditions may be attributed to photosynthesis impairment and the induction of oxidative stress.

Ions Analyses

We studied the effects of increasing levels of salinity on ion uptake in *Marrubium vulgare*. Salt treatment significantly reduced the uptake of cationic elements K^+, Ca^{2+}, Fe^{2+}, and Zn^{2+} with a dose-dependent accumulation of Na^+ in the aerial part (Table 2). However, a high accumulation of Na^+ in *Marrubium vulgare* caused a nutriment unbalance that was at the origin of the depressive effect on plant growth (Fig. 1.) and could result from an antagonistic effect between Na^+ in one part and K^+, Ca^{2+}, Fe^{2+}, and Zn^{2+} in the other part. In the same way, [36] reported that salinity induced a strong increase of Na^+ content in NaCl-tolerant cell lines. Consequently, the total charges must be balanced in the cells by the decrease of the others elements. The decline in ion accumulation and their transport selectivity may serve as an important physiological trait of adapting to NaCl stress, which has been well documented in *Ocimum basilicum* L. [28], in *Solanum lycopersicum* [37], *Oryza sativa* L [38], and *Solanum nigrum* [39].

Marrubiin Content

As marrubiin is the major constituent of *M. vulgare*, and the active principle responsible for the medicinal properties of the plant, the effect of salt stress on the production of this compound was investigated.

The identity of isolated marrubiin was confirmed by FTIR, ^{1}H, and ^{13}C NMR [20]. Analysis of proton spectra (Fig. 3) showed three well-separated peaks at 6.29, 7.25, and 7.38 ppm, corresponding to protons H-15, H-14, and H-16, respectively. The intensity of the peak at 7.38 ppm was used to quantify the amount of marrubiin in the acetone extracts of dry materials.

Marrubiin content decreased gradually with increased NaCl concentrations. The averaged values were 1.38, 0.93, 0.62, and 0.34 mg/g DW for concentrations of 0, 25, 50, and 100 mM NaCl, respectively. The obtained values of detected marrubiin in different samples were relatively low. We can explain this by the old age of harvested plants (five months). However, the accumulation of furanic labdane diterpenes was previously investigated in plantlets of *M. vulgare* during the first three months following germination in order to get information on the time when accumulation starts; the authors concluded that the accumulation of furanic labdane diterpenes in older leaves was rather low. The amount of marrubiin found in leaves of 12-week-old plantlets of *M.vulgare* was 200 µg/FW [40]. As a conclusion, the quantitative variation of marrubiin

Table 1. Variation of fresh weight (FW), height, chlorophyll (a), chlorophyll (b) and total chlorophyll content of plants subjected to water with different NaCl concentrations (0, 25, 50, 100 mM) during 5 months.

NaCl (mM)	Fresh weight (g)	Height (cm)	Chlorophyll (a) (mg/gFW)	Chlorophyll (b) (mg/gFW)	Total chlorophyll (mg/gFW)
0	236.24±6.26^{a}	39± 4.58^{a}	1.00±0.03^{a}	0.41±0.01^{a}	1.42±0.02^{a}
25	115.72±4.12^{b}	28±2.51^{b}	0.63 ±0.01^{b}	0.34±0.00^{b}	0.98±0.01^{b}
50	95.17±1.97^{c}	25±7.51^{b}	0.85±0.02^{b}	0.35±0.00^{b}	1.19±0.02^{b}
100	20.85±2.07^{d}	9±5.29^{c}	0.41±0.00^{c}	0.08±0.01^{c}	0.49±0.03^{c}

Averages ± standard deviation were obtained from three different experiments. Data with the same letter are not different according to ANOVA (analysis of variance) ($p < 0.05$). The results are sorted in decreasing order: a > b > c > d.

Table 2. Variation of Na^+, K^+, Ca^{2+}, Fe^{2+}, Zn^{2+} contents (µg/g DW) of *M. vulgare* subjected to different NaCl concentrations (0, 25, 50 and 100 mM) during 5 months.

Treatment (NaCl)	Na+	K^+	Ca^{2+}	Fe^{2+}	Zn^{2+}
0 mM	1230±10^c	28700±290^a	22600±670^a	690±10^a	70±3^a
25 mM	2130±30^c	27400±120^a	20900±20^b	530±10^b	50±3^b
50 mM	6160±490^b	23130±20^{ab}	19700±40^c	460±30^{bc}	40±5^c
100 mM	16230±220^a	17630±580^b	17900±50^d	370±40^c	40±5^c

Averages ± standard deviation were obtained from three replicates. Data with the same letter are not different according to ANOVA (analysis of variance) ($p<0.05$); DW, Dry weight.

content can be attributed to several factors, e.g., seasons, environment, genes, varieties, origin, methods of extraction, and plant part.

In our present work, the results suggest that the synthesis/accumulation of marrubiin in *M. vulgare* is particularly sensible to salt stress.

The decline of diterpenes content in response to salinity has been previously reported in clary sage (*Salvia scalrea*), where a significant decrease of manoyl oxide and phytol was observed in hydroponic culture containing 25, 50, and 75 mM NaCl [41]. In our case, it seems that the reduction in marrubiin content is likely due to the inhibition of its correspondent biosynthesis enzymes, namely diterpenes synthases [41] in response to salinity. Another possible explanation is that the decline of marrubiin could be linked with the accelerated senescence induced by salt stress as reported in *Cistus creticus* [43]. By using transcriptomic analysis, these authors have successfully proved that young leaves accumulated more labdane-diterpenes than older leaves, and such a trend is highly influenced by abiotic stress [44].

Total Phenolic Content

Compared with the control, salt-treated plants exhibit lower values of TPC. According to the literature, the effect of salinity on TPC has been found to depend on the species and the stress intensity, our results are in conformity with the study about *Nigella sativa*, salt treatment was found to affect phenol content, which decreased by 30, 54, and 61% in response to 20, 40, and 60 mM NaCl [6]. An earlier study showed that the exposure of *Coriandrum sativum* causes a significant decrease in polyphenol accumulation occurring in about 37%, 43%, and 66%, respectively, at 25, 50, and 75 mM, with respect to the control NaCl [44]. These author's reports concluded the salt sensitivity of studied plants. According to [31], the drastic effect of salinity (75 mM) on TPC of *Salvia sclarea* L. may be due to a less efficient ROS as a result of an imbalance between ROS and antioxidant formation, leading to the installation of the oxidative stress. However, this trend is contradictory to that observed in cucumber [46], *Carthamus tinctorius* [47, 48], *Salvia officinalis* [30], and *Origanum majorana*

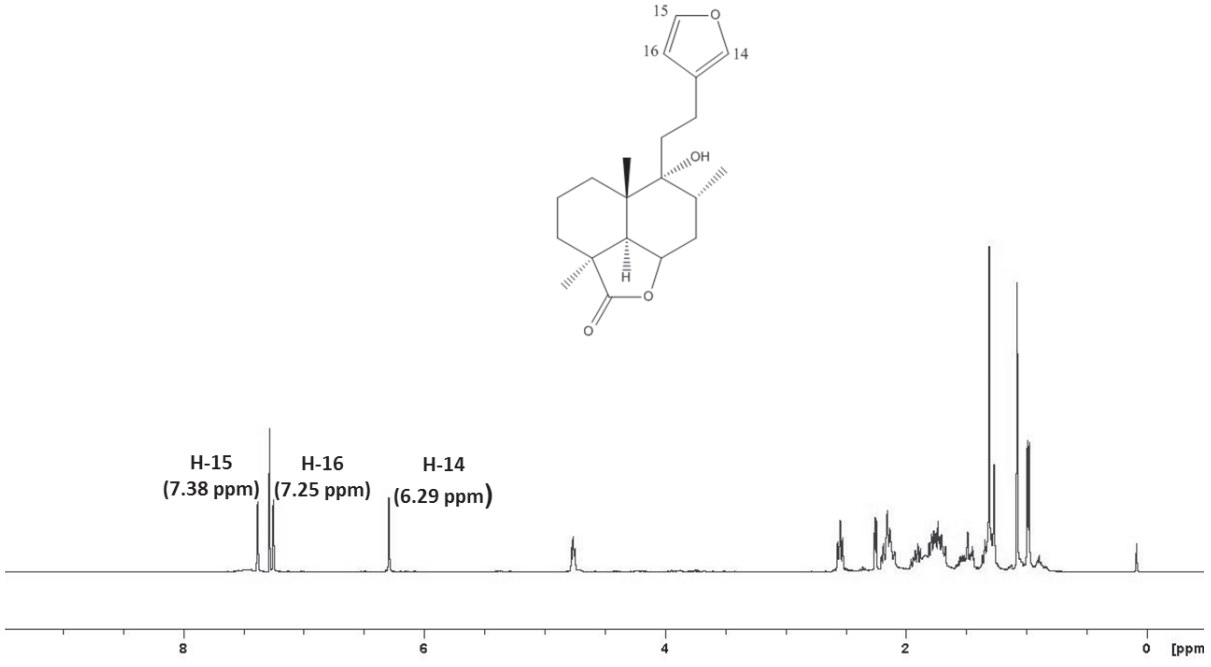

Fig. 3. ^{1}H-NMR spectra of marrubiin.

Table 3. Variation of methanol extract weight (EW, mg/gFW), total phenolic content (TPC, mg GAE/gFW) and antioxidant activity of *M. vulgare* subjected to different NaCl concentrations (0, 25, 50 and 100 mM) during 5 months. Antioxidant activity was evaluated by the reducing power assay, the DPPH test and bleaching of beta carotene assay.

Salinity (NaCl, mM)	Methanol extract (mg/g FW)	TPC (mg GAE/gFW)	Reducing power Fe^{3+}/Fe^{2+} (mg Asc AE/gEW)	DPPH scavenging (EC_{50} µg/mL)	Bleaching of beta carotene (%) *
0	41.9	3.7±0.01[a]	96.89±3.24[a]	499.58 ±2.45[c]	n.a.
25	46.9	2.1±0.03[b]	3.05±0.40[c]	807.38±8.33[b]	15.92
50	14.9	0.8±0.00[b]	58.95±0.23[b]	857.26±0.22[a]	27.51
100	22.8	1.2±0.00[b]	n.a	199.86±13.1[d]	40.42

Averages ± standard deviation were obtained from three different experiments. Data with the same letter are not different according to ANOVA (analysis of variance) ($p<0.05$). n.a : no activity (≤ 5%); FW, Fresh weight; GAE, gallic acid equivalents; EW, Extract weight; Asc AE equivalents, mg ascorbic acid/g sample.
*concentration tested: 1.6 $mg.mL^{-1}$.

[49]. In the present study, the decrease in TPC could be ascribed to decreased activity of phenylalanine ammonia lyase (PAL: the first enzyme of the phenylpropanoid pathway that catalyzes the elimination of NH_3 from L-phenylalanine to produce *trans*-cinnamate) probably due to the accumulation of toxic levels of Na^+ and Cl^-. Supporting information is provided by [45], who reported that decreased phenol accumulation was associated with lower PAL activity in wheat. The susceptibility of the phenylpropanoid metabolism to salinity justifies our previous conclusion about the sensitivity of *M. vulgare* to salt stress.

To test whether the changes in TPC content could influence the antioxidant capacity of *M. vulgare* extracts, three complementary assays were performed.

Antioxidant Activity

Data depicted in Table 3 show that all methanol extracts have the capacity to scavenge the DPPH radical. The most active extracts was those derived from plants treated with 100 mM NaCl (EC_{50} = 199.86 ±13.1 µg/mL), followed by those issued from untreated samples (EC_{50} = 499.58±2.45 µg/mL). Salt-treated plants with 25 and 50 mM NaCl showed the lowest activity with an EC_{50} of 807.38±8.33 and 857.26±0.22 µg/mL, respectively. These data clearly show that the radical-scavenging activity of methanol extract was not directly linked to the TPC content. The equivocal correlation between the TPC and antioxidant activity can be explained in several ways: In fact, the TPC fraction does not necessarily incorporate all the antioxidants. The synergistic interactions between antioxidants in the extract make the antioxidant power not only dependent on the concentration but also in the structure and the nature of antioxidants. Besides, the antioxidant process is very complex, depending on several modes of polyphenol interaction that can participate to different degrees because of their different ratio among single classes or subclasses [50]. Indeed, in the report of [51], who tested the antioxidant activity of individual phenolic compounds, flavan-3-ols possessed the highest radical scavenging activity while the mechanism of antioxidant activity depended on specific structures, e.g., the number of hydroxyl groups and the presence of the -CH=CH-COOH group. Therefore, The high anti-radical activity observed in plants treated with 100 mM was presumably also due to the implication of antioxidant enzymes (i.e., SOD, CAT, POD, and APX) as well as antioxidant metabolites (i.e., such ascorbic acid, reduced glutathione, carotenoids, tocopherols, alkaloids, and non-protein amino acids [52] or to particular phenolic compounds induced by salt treatment. The decrease in the DPPH-radical scavenging effect was similar to that observed in *Coriandrum staivum* [44]. Thus, it has been found under saline conditions that the scavenging activity was strongly diminished, the IC50 values increased by 116%, 129%, and 188%, respectively, at 25, 50, and 75 mM NaCl in comparison to the control. The authors suggest that an imbalance between reactive oxygen species (ROS) generation and scavenging systems might have occurred in *Coriandrum staivum* under salt treatment.

We also evaluated the reducing power of methanol extract (Table 3). As shown, the methanol extracts from untreated plants present the highest reducing power and that effect decreased with increasing salinity. The decrease in the reducing power of the extract can probably be due to the decrease in the phenol content (Table 3). However, there are some drawbacks regarding this test since it is assumed that all antioxidant compounds (reductants) able to produce the Fe(III)/Fe(II) complex are also able to reduce ROS and NOS (reactive nitrogen species). This statement is true for ascorbic acid and many phenols, but it does not apply to all compounds, such as, for example, gluthatione – an important antioxidant *in vivo* but unable to efficiently reduce Fe(III).

By using the β-carotene/linoleic acid model system, only extracts from salt-treated plants were found to be active (Table 3; Fig. 4). Plants treated with 100 mM NaCl present the highest activity (40.42%), followed by treatment with 50 (27.51%) and 25 mM NaCl (15.92%).

The strong antioxidant activity observed in plants treated with 100 mM NaCl might be due to factors other than the amount of phenolic compounds [53]. These authors reported a significant correlation between the total

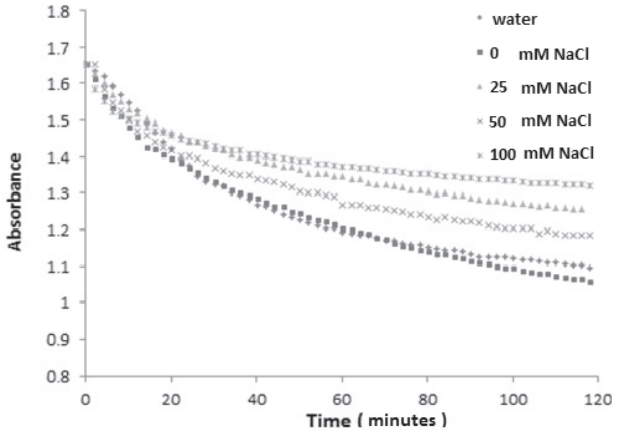

Fig. 4. Kinetic behavior of β-carotene bleaching of methanolic extract from *M.vulgare* at 1.6 mg.mL^{-1}.
Averages ± standard deviation were obtained from three different experiments. Data with the same letter are not different according to ANOVA (analysis of variance) ($p<0.05$). n.a: no activity (≤5%); EW, extract weight; GAE, gallic acid equivalents; Asc AE equivalents, mg ascorbic acid/g sample.
*concentration tested: 1.6 mg.mL^{-1}

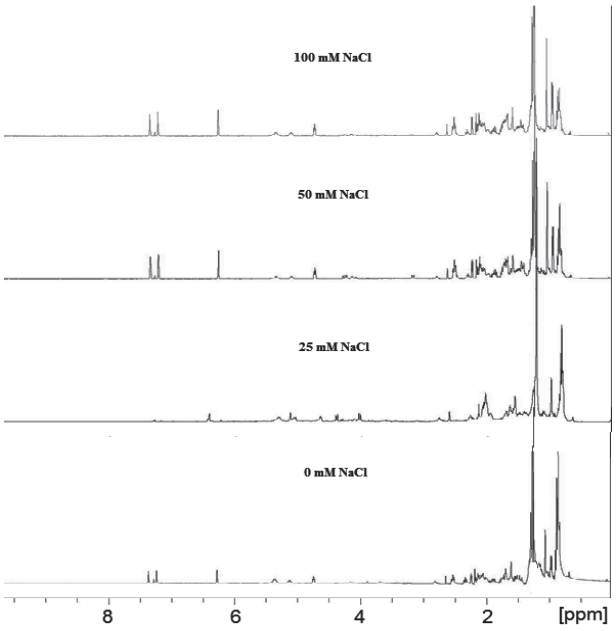

Fig. 5. ^{1}H-NMR spectra of petroleum ether extracts of *M.vulgare* subjected to different concentrations of NaCl (0, 25, 50, and 100 mM) after five months of treatment.

phenolic and antioxidant capacities for flaxseed and cereals, but not for the anthocyanin-rich materials and medicinal plants, which is similar to our results. Besides, another interesting phenomenon called the "polar paradox" has been reported [54]. It is observed that lipophilic antioxidants are more effective in oil-in-water emulsion systems than in bulk oil, while the hydrophilic antioxidants are more effective in bulk oil systems. This behaviour is explained by the stronger affinity of the non-polar antioxidants to the oil-water interface of the emulsion and the high affinity of the polar antioxidants to the air-oil interface. As a result, non-polar antioxidants concentrate in the interface of the oil droplets, ensuring high protection of the emulsion itself and, on the other hand, polar antioxidants remaining dissolved in the aqueous phase are more diluted and hence less effective. This phenomenon can explain the increased antioxidant activity evaluated by this test that is opposite to the evaluation of total phenol content.

To test the presence of lipophilic compounds, the petroleum ether extracts were analyzed by ^{1}HNMR.

^{1}HNMR Estimation of Fatty Acid Content and Composition

^{1}HNMR spectra of petroleum ether extract of *M. vulgare* was investigated in the region of 5.8 ppm to 0.5 ppm (Fig. 5). These spectra are very complex since they correspond to a mixture of lipid compounds. However, some information can be obtained from the careful investigation of these spectra. According to [25], a multiplet at 5.40 and 5.26 ppm observed in the spectra of oils can be assigned to the hydrogen atoms linked to the double bonds of acyl groups of glycerides, and in the region 5.26 and 5.20 ppm to the proton linked to carbon 2 of glycerol of a glyceryl group. In the spectra of the petroleum ether extracts these two peaks had merged in a multiplet. The center of this multiplet moved from 5.31 to 5.37 ppm with increasing salt stress, which can be explained by the disappearance of triglycerides and the appearance of increasing amounts of free fatty acids.

Additional confirmation was obtained by a similar decrease of the intensity of the multiplet at 4.32 and 4.10 ppm, corresponding to the methylene of glycerol in triglycerides. In fact, the reduced intensity of this peak when salinity increases indicates that the ester function disappears. This information was further confirmed by a similar change at 2.38 ppm and 2.27 ppm, corresponding to the alpha-methylene to the carboxylate of the ester or of the free fatty acid. According to [21], there is a small difference between these protons, namely that those from the ester produce a triplet at a higher field (lower ppm) than those from free fatty acids. The spectra of a mixture of triglycerides and free fatty acids show a multiplet corresponding to the superposition of these peaks. In the observed spectra the intensity of the signal at 2.27 ppm moves respectively to 2.31, 2.33, and 2.34 ppm with increasing salinity, which in our opinion is due to increasing amounts of free fatty acids in the extract.

Regarding the composition of the acyl groups, a ratio between the intensity of the methylene protons at 2.27 ppm present in all acyl groups, and those at 2.83 ppm belonging only to the polyunsaturated ones [25] can be established. This ratio is similar in the control and the plants treated with 25 mM and 50 mM NaCl, indicating that apparently there is no appreciable change in fatty acid composition. However, at the highest dose of salt, NaCl 100 mM, the

presence of unsaturated acids dramatically decreases, indicating a desperate effort of the plant to maintain its integrity [55].

Conclusions

As a final conclusion it can be said that salt stress decreases growth and biomass of *M. vulgare* L. areal parts as well as total phenolic content. Furthermore, environmental stresses can significantly enhance some antioxidant properties such as radical scavenging capacity and inhibition of lipid peroxidation.

Therefore, in our experiment we thought that the increase of antioxidant activities under saline conditions may contribute to the ability of the plant to survive in adverse environmental stress as salinity.

The production of marrubiin, the main bioactive compound extracted from *M. vulgare,* is greatly affected by salinity. The increase in salt concentration to 25 mM has the consequence of lowering the production of marrubiin by about 23%. Doubling the NaCl concentration to 50 mM, we observed an important decrease of this important bioactive compound content of about 50% of the control plants.

This information can be useful for pharmaceutical companies interested in farming *M. vulgare* as a phytomedicine or local healers, since this environmental stress will affect the medicinal properties of the plant. Nevertheless, taking into account this information, *M. vulgare* can be an accessible fragrances in flavoring additives and beverages. Therefore, further investigation can be important to know the development of new chemotypes at different salt levels, which could be considered as a valuable source for functional foods, nutraceuticals, cosmetics, and pharmaceutical industries.

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