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Research article

Qualitative and quantitative analysis of diosmin content of hyssop (*Hyssopus officinalis*) in response to salinity stress

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ABSTRACT

Hyssop (*Hyssopus officinalis* L.) is a perennial subshrub, which is distributed across the eastern Mediterranean region to central Asia. One of the most important bioactive compounds of hyssop is diosmin, a flavone glycoside of diosmetin, with application in the field of cardiovascular therapy. Salinity as one of the most essential environmental stress factors is able to alter secondary metabolite content in plants. Therefore, we aimed to investigate the effect of salinity on the levels of total flavonoid content and diosmin in hyssop. Accordingly, salinity stress was imposed by watering plants with four different concentrations of sodium chloride (NaCl) (50, 100, 150 and 200 mM) for 4 weeks. High-performance liquid chromatography (HPLC) method was used for purification of diosmin from dried leaves and measurement of it in dried shoots. Nuclear magnetic resonance (NMR) spectroscopy was applied for determination of the structure of diosmin. The obtained results showed that high salinity levels lead to a higher amount of total flavonoid and diosmin content in treated plants. Although alteration in diosmin content was not significant in treatments up to 100 mM NaCl, higher amounts of diosmin were observed in 150 and 200 mM NaCl salinity levels. We concluded that the contents of total flavonoid and diosmin were significantly elevated after exposure of hyssop plants to salt stress conditions.

1. Introduction

Hyssop (*Hyssopus officinalis* L.) as a perennial subshrub of the family Lamiaceae is distributed in the eastern Mediterranean to central Asia [1]. This popular medicinal herb is considered as a carminative, tonic, antiseptic, expectorant and is employed as a remedy in congestion, lung complaints and cardiovascular disorders [1, 2]. The essential oils extracted from hyssop shoots possess a unique aroma and is widely used in the food, pharmaceutical and cosmetics industries [3, 4]. Different polyphenolic compounds were identified in this plant including flavonoids and their glycoside derivatives such as quercetin, apigenin, diosmin, luteolin [1]. One of the main bioactive flavonoids of hyssop is diosmin. Nowadays, diosmin has found many applications as anti-inflammatory agents in the clinical treatment of cardiovascular diseases such as venous stasis, vein wall remodeling, chronic venous insufficiency, lymphedema and varicose veins [5].

H. officinalis is known as a typical xerophyte and is well adapted to drought and salinity conditions [6]. Salinity is one of the most essential issues that modifies the world's farmlands, and is considered to be the main factor that limits the dispersal of plants in their natural habitats [7]. Salinity levels in soil may induce a wide range of damages at the cellular and whole plant levels, and thus limit the growth and yield of plants [8]. In fact, the saline condition has detrimental impacts on physiological and biochemical parameters of plants through decreases in soil water potential, impairment of mineral nutrition, ionic toxicity and secondary stress such as the production of reactive oxygen species (ROS) [9].

In aromatic plants such as species belonging to the family Lamiaceae, salinity may result in substantial changes in the compositions of some metabolites and yields [10, 11]. For example, it was reported that NaCl treatments led to significant increases in the level of total phenols and flavonoids in *Thymus vulgaris* and *Thymus daenensis* [12]. Further, exposure to 150 mM NaCl increased the content of total polyphenols in *T. vulgaris* [13]. Definitely, salinity is accompanied by oxidative stress owing to the generation of ROS. Under moderate salt stress, synthesis of some metabolites may rise for inhibition of the effects of ROS in plant cells. In view of that, different levels of salinity stress raised flavonoid and phenolic contents as well as antioxidant activity in *Portulaca oleracea* and *Cichorium spinosum* [14].

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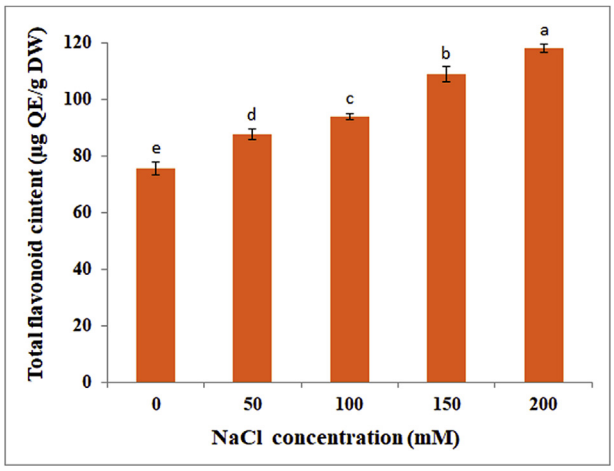

Figure 1. The effect of different levels of NaCl (0, 50, 100, 150 and 200 mM) on the total flavonoid content in hyssop.

The variation of essential oil composition of hyssop in the flowering stage was reported as a consequence of saline condition [6]. However, there is no report on the effect of salinity on the content of bioactive flavonoids in this medicinal herb. Thus, the main aim of the current work was to investigate the changes in the diosmin content in hyssop under a broad range of saline stress. The findings of this work provide a framework for further examination of flavonoid contents in this plant under various environmental conditions.

2. Material and methods

2.1. Plant material and stress conditions

Seeds of *H. officinalis* were purchased from Pakan-Bazr Isafahan, Iran. Seeds were sown in sterile perlite:sand (1:3) pots and placed at 4 °C for 4 days for stratification. Plants were grown in a modified 10% Hoagland basal medium (10 HM) in a greenhouse at a 25/22 °*C day*/night temperature regimes, at a 16/8 h day/night cycle and a relative humidity of 60%. Plants were irrigated twice a week with deionized water during the experiment. Salinity stress was imposed in seed germination stage with four levels of NaCl concentrations including 50, 100, 150 and 200 mM. Concurrently, control groups of plants were grown without saline treatment. Induction of salinity was continued twice weekly for one month. The shoots of treated hyssop plants were collected and analyzed to determine the changes in the total flavonoid and diosmin content at different salinity levels.

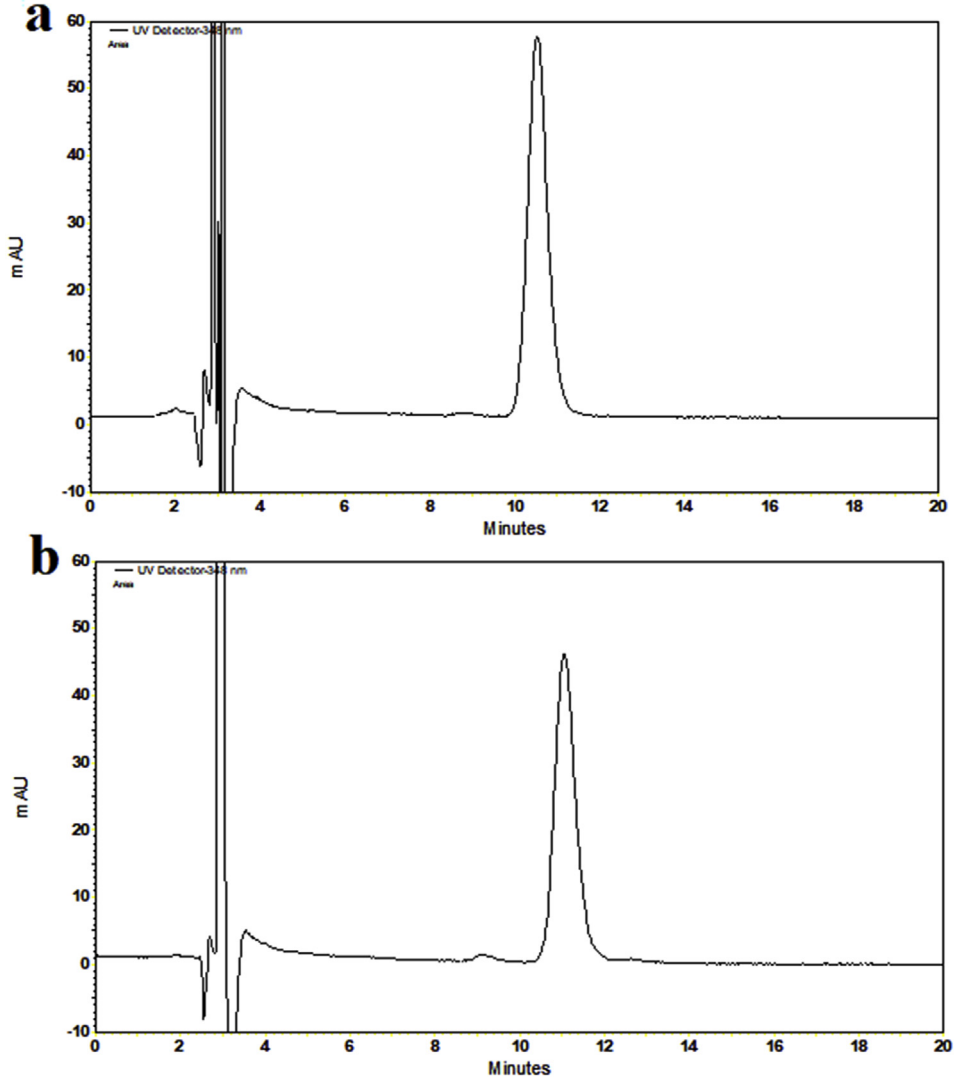

Figure 2. The chromatograms of (a) standard diosmin and (b) purified diosmin in 348 nm.

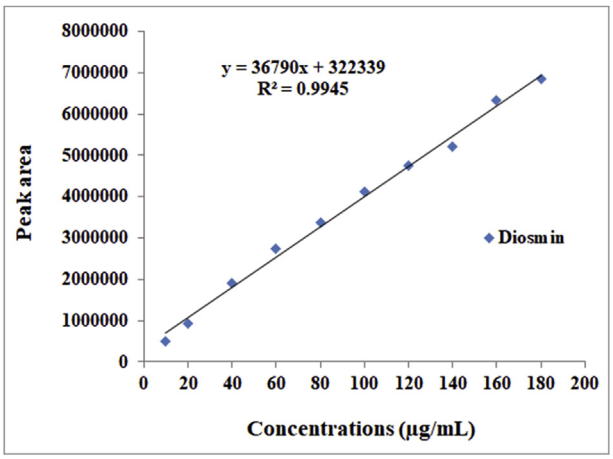

Figure 3. The standard curve of diosmin.

2.2. Determination of total flavonoid content

The total flavonoid content of the samples was determined using aluminum chloride colorimetric method [15]. 0.1 g of ground shoot was extracted with 5 mL of 80% ethanol for 20 min in an ultrasonic bath at room temperature. The extract was separated by centrifugation at 3000 rpm for 15 min. The extraction process was repeated three times on a single sample and the obtained extracts were mixed together. 0.5 mL of hydroalcoholic shoot extract was mixed with 4.5 mL of distilled water in a test tube. Then, 0.5 mL of 5% aqueous $NaNO_2$ solution was added and the mixture was maintained for 5 min at room temperature. Afterwards, 0.5 mL of 10% aqueous $AlCl_3$ solution was added and after 6 min, 4 mL of 1 M aqueous NaOH solution was added. Finally, after 15 min, the absorbance of the solution was measured at 510 nm by a spectrophotometer (PD-303 UV, APEL, Japan) at room temperature. The measurements were repeated in triplicate. The total flavonoid content was expressed with respect to quercetin standard curve as µg quercetin equivalent per gram dry weight (µg QE/g DW). The results were derived from the calibration curve ($y = 0.740x - 0.005$, $R^2 = 0.987$) of quercetin (concentration range: 100–1400 µg/mL).

2.3. Isolation and purification of diosmin

Standard diosmin (3′,5,7-trihydroxy-4′-methoxyflavone 7-rutinoside) with a purity ≥90%, and all solvents of HPLC grade were purchased from Sigma-Aldrich Chemie GmbH, USA. The ultrapure water used for mobile phase preparation was obtained by means of a Milli-Q system (Millipore, Bedford, MA, USA). Also, the dried leaves of *H. officinalis* were purchased from Pakan-Bazr Isafahan, Iran. The dried ground materials (50 g hyssop leaves) were pre-extracted by Soxhlet apparatus with 750 mL of n-hexane, chloroform, absolute ethanol and acetone, respectively, for the elimination of undesirable lipophilic compounds. After removal of the solvent, dimethyl sulfoxide (DMSO) was added to the dried plant materials and the mixture was stirred at room temperature for 12 h. For precipitation of diosmin, the filtered extract was diluted with ultrapure water (1:10) at 4 °C prior to the centrifugation step. The obtained sediments were washed several times with ultrapure water and centrifuged at 3000 rpm for 15 min. The acquired sediments were dissolved in a mixture of DMSO-methanol (1:1, v/v) and applied for purification by a preparative HPLC system [16].

Purification of diosmin was performed using a Knauer preparative HPLC system equipped with a ODS (C18) column (250 × 21.2 mm, 10 µm). The extracts were filtered through a nylon membrane with a pore size of 0.45 µm before injection into the HPLC apparatus. The injection volume was 200 µL and the mobile phase was a mixture of methanol-water (1:1, v/v). The flow rate was 8 mL/min and the analytes were monitored at 348 nm. All chromatographic operations were carried out at ambient temperature.

2.4. Identification of diosmin

Ultraviolet–visible (UV) spectroscopy and one-dimensional nuclear magnetic resonance (NMR) spectroscopy were used to identify diosmin. The UV spectrum of the purified compound was recorded in methanol using SHIMADZU UV-2450 UV-Visible Spectrophotometer in the range of 190–400 nm. Also, the ^1H NMR and ^{13}C NMR spectra of purified sample were recorded by a NMR spectrometer (Bruker Avance III 300 MHz, Germany) in hexadeuterodimethyl sulfoxide (DMSO-d_6, 99.9 atom % D, ACROS Organics, USA) for determining the molecular structure. ^1H NMR and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively [17].

2.5. Determination of diosmin content by HPLC

To determine the amount of diosmin, 500 mg of samples were dissolved in 5 mL of 80% ethanol and sonicated for 20 min at 20°C. The resulted mixture was centrifuged at 3000 rpm for 15 min and the supernatant was separated. The extraction procedure was repeated twice using the remaining plant sediment. The obtained supernatants were combined and 20 µL of the obtained extract was injected into the analytical HPLC system equipped with a C18 analytical column (250 × 4.6 mm, 5 µm). The mobile phase was a mixture of methanol-water (1:1, v/v). The flow rate was 0.8 mL/min and effluents were monitored at the wavelength of 348 nm. Each sample was injected three times and the amount of diosmin was calculated regarding the area under the curve. To prepare a calibration curve for HPLC quantification, serial concentrations of standard diosmin (10–180 µg/mL in methanol-DMSO (1:1, v/v)) were used [18, 19]. The calibration curve of diosmin was plotted according to the area under the curve versus the concentration.

2.6. Statistical analysis

The experiments were designed as a completely randomized design (CRD) with three replicates for each treatment and each replicate was represented by 8 seedlings. Treatment means were compared by the analysis of variance (ANOVA) using SPSS (version 23, for Windows; SPSS

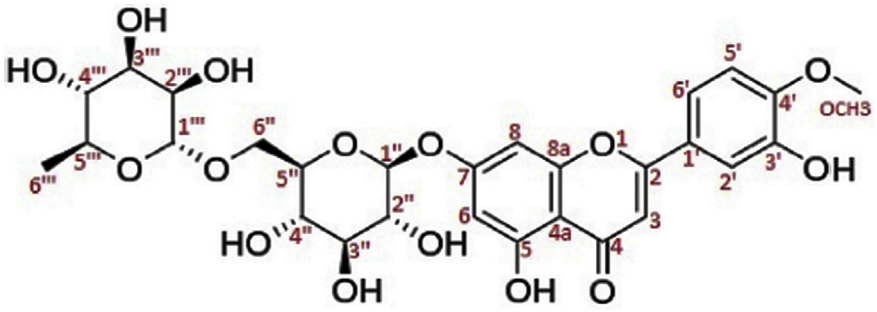

Figure 4. The chemical structure of diosmin.

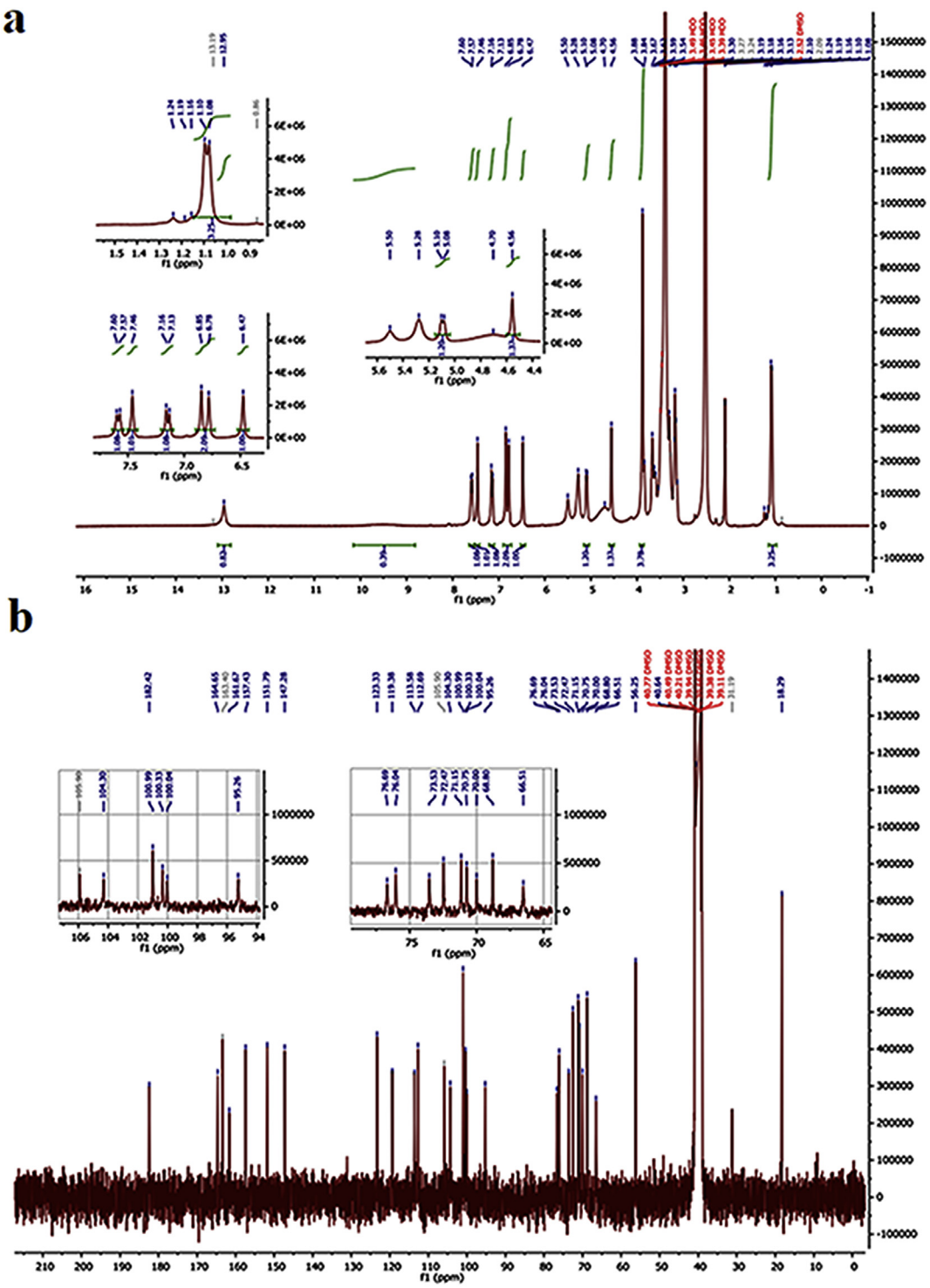

Figure 5. The NMR spectra of diosmin, a) proton NMR (^{1}H NMR) spectrum (300 MHz, DMSO-d_6) and b) carbon NMR (^{13}C NMR) spectrum (75 MHz, DMSO-d_6).

Inc., Chicago, IL, USA). Least significant difference (LSD) between treatment means was calculated at the 5% probability level.

3. Results and discussion

3.1. Effect of salinity on total flavonoid content

The flavonoids are a group of secondary metabolites, which have a strategic function in neutralizing oxidative damages encouraged by abiotic stresses like salinity. As shown in Figure 1, the total flavonoid content of hyssop leaves was measured to be 75.44 μg QE/g DW in the control plants. After exposure to 50 mM NaCl, a significant upsurge in total flavonoid content was observed, which was 1.2 times higher than that of the control plants. After treatment with high salinity levels (150 and 200 mM), the total flavonoid contents were 44% and 56% higher than control samples. Therefore, it could be established that total flavonoid accumulation in hyssop leaves was enhanced by the increasing salinity levels from 50 to 200 mM.

In line with our results, in previous studies, the content of phenolic and flavonoid compounds were increased in *Cichorium spinosum*,

Table 1. Analysis of variance in terms of different salinity level stress in two harvests on the amount of diosmin in hyssop plant.

Source of changes	Degree of freedom	Average of squares
		Diosmin amount
Harvest stage	1	857523.07**
Salinity stress	4	1271472.45**
Harvest stage× Salinity stress	4	1100925.57**
Experimental error	20	12320/52
Variation coefficient percentage		6/59

** Means a significant difference at 1% probability level.

Fagopyrum esculentum, *Salvia mirzayanii*, *Portulaca oleracea*, *Carthamus tinctorius*, *Amaranthus tricolor*, *Thymus daenensis* and *Thymus vulgaris* under NaCl stress [11, 12, 14, 20, 21, 22, 23].

3.2. Identification and quantification of diosmin

The HPLC chromatograms obtained for the standard diosmin and diosmin purified from hyssop leaves are presented in Figure 2. A comparison of the chromatograms revealed that diosmin isolated from plant material has a sufficient purity. Based on the obtained data, the calibration curve of diosmin was linear in the 10–180 μg/mL range with a correlation coefficient of 0.9945 (Figure 3). As shown in the related chromatogram, a good separation was achieved at the retention time of 11 min for diosmin from hyssop leaves during the working day at a wavelength of 348 nm (Figure 2).

The chemical structure of the compound purified by preparative HPLC was elucidated using the NMR technique, which confirms the structure of diosmin (Figure 4) [17]. The NMR spectra of diosmin are shown in Figure 5. The information obtained from spectroscopic studies of the purified diosmin is summarized as follows: UV λ_{max} (Methanol): 348 nm. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.95*s* (1H, OH-5), 9.46*s* (1H, OH-3′), 7.59br*d* (1H, J = 9.03 Hz, H-6′), 7.46bs (1H, H-2′), 7.15br*d* (1H, J = 9.03 Hz, H-5′), 6.85*s* (1H, H-3), 6.78br*s* (1H, H-8), 6.47br*s* (1H, H-6), 5.09*d* (1H, J = 6.02 Hz, H-1″), 4.56br*s* (1H, H-1‴), 3.88*s* (3H, OCH_3-4′), 3.10–3.90 (carbohydrate protons, H-2‴ H-6‴, H-2‴ H-5‴), 1.09*d* (3H, J = 6.02 Hz, H-6‴); ^{13}C NMR (75 MHz, DMSO-d_6): δ 182.42 (C-4), 164.65 (C-2), 163.40 (C-7), 161.67 (C-5), 157.43 (C-8a), 151.79 (C-4′), 147.28 (C-3′), 123.33 (C-1′), 119.38 (C-6′), 113.58 (C-2′), 112.69 (C-5′), 105.90 (C-4a), 104.30 (C-3), 100.99 (C-1‴), 100.33 (C-6), 100.04 (C-1″), 95.26 (C-8), 76.69 (C-3‴), 76.04 (C-5‴), 73.53 (C-2‴), 72.47 (C-4‴), 71.15 (C-4″), 70.75 (C-2″), 70.00 (C-3″), 68.80 (C-5″), 66.51 (C-6″), 18.29 (C-6‴).

Based on our finding, isolation and purification of diosmin from hyssop leaves by preparative HPLC was very effective with a purity of 98.4% (Figure 2b). These outcomes are in agreement with the previous reports that HPLC techniques could be successfully applied for purification of diosmin [18]. Similar to our work, a HPLC-based technique has been previously used for the study of flavonoids in a hydroalcoholic extract of whole plant of *Bassia eriophora* (Chenopodiaceae) [24]. A preparative HPLC method was applied and the isolated compounds were characterized by NMR. Accordingly, five flavonoids, including diosmin, were characterized [24]. However, in the present study, the purified diosmin in our laboratory was used for the next stages of the study to analyze diosmin in hyssop plants under salinity stress.

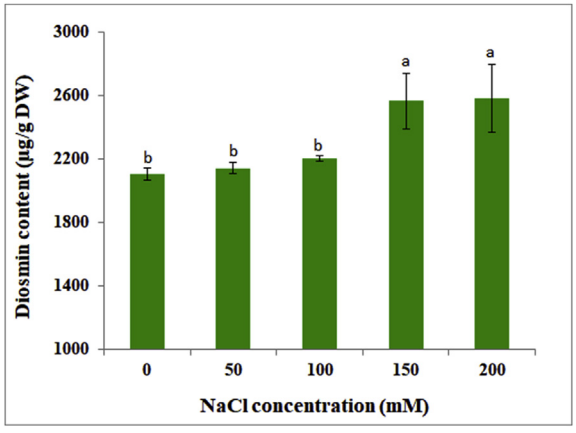

Figure 6. The effect of different levels (0, 50, 100, 150 and 200 mM) of salinity (NaCl) on diosmin content in hyssop.

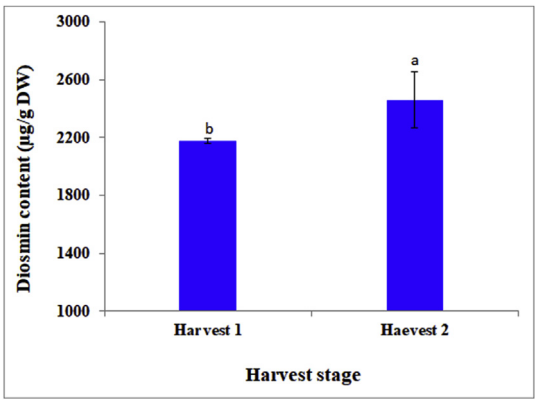

Figure 7. Comparison of simple effect of salinity levels on diosmin content of hyssop plants at the first and second harvest (before and after salt stress).

3.3. Influence of salinity levels on the diosmin content

The effect of different salinity levels on the diosmin content of shoots of hyssop in the vegetative stage was assessed using the analytical HPLC method. The analysis of variance in terms of the content of diosmin revealed a significant difference at the level of 0.001 between the treatment groups and harvest stages in the shoots of the hyssop plant under the salinity stress (Table 1).

Our data showed that the salinity stress results in a significant increase in the amount of diosmin in the shoots of hyssop plant. The diosmin content was enhanced by the increasing concentration of NaCl and the highest amount of diosmin was achieved after treatment of plants with 150 and 200 mM of NaCl (2581.12 μg/g DW) (Figure 6). Comparing the diosmin content in shoots of treated plant samples before and after the treatments revealed a significant increase in the amount of diosmin after the salt stress by 1.13 fold in comparison to the first harvest before the salt stress as shown in Figure 7.

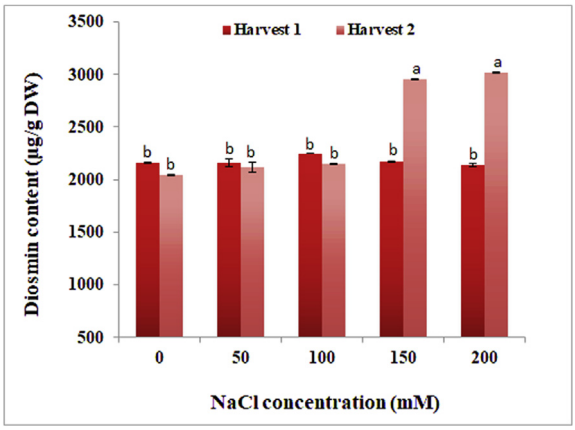

Figure 8. Comparison of the interaction levels of salinity stress (0, 50, 100, 150, 200 mM NaCl) and harvesting stages (first and second harvests respectively before and after salt stress) on the amount of diosmin in hyssop plant.

To determine the effects of salinity levels on the diosmin content before and after the salinity treatments, the amount of diosmin has been evaluated in samples before and after NaCl treatments. In low and moderate salt levels, the amount of diosmin was not increased after exposure to saline condition, while at the higher levels (150 and 200 mM) the amount of diosmin was significantly increased (Figure 8). These results showed that the effect of NaCl on diosmin content is dose-dependent. It is well known that salinity stress may upsurge the level of secondary metabolites. It was reported that the content of total polyphenols, total flavonoids, β-carotene, vitamin C, and total antioxidant activity of *Amaranthus tricolor* (genotype VA13) were significantly affected by salinity stress [23]. In this study, the contents of flavonoids isoquercetin and rutin, and also *m*-coumaric acid were increased in *A. tricolor* with the harshness of salinity stress [23], which confirmed the obtained results in our study.

Obviously, secondary metabolites accumulation protected safflower plants against salt-stress conditions [25]. Likewise, increasing salinity enhanced lipids content, carotenoids, and antioxidant activity in *Amphora subtropica* [26]. Salt stress was also resulted in elevated contents of gossypol, flavonoids and tannins in cotton after salt stress [27]. The changes in plant growth, antioxidant capacity, and volatile exudates were studied in *Schizonepeta tenuifolia* exposed to salt stress [28]. The results indicated that its dry biomass was reduced by salt treatments and the contents of antioxidants, including phenolics and flavonoids, increased at low (25 mM) or moderate (50 mM) levels, but declined at severe (75 and 100 mM) levels. To sum up, salt stress significantly influenced the growth and secondary metabolism of *S. tenuifolia* [28].

In line with our results, an increase in essential oil content of different plant species has been also reported under salt stress [29]. In addition, the influence of increased NaCl concentration in the culture medium on the essential oil composition and phenolics contents in the leaves of *Salvia officinalis* was investigated [30]. Moreover, the concentrations and total contents of diterpenes (carnosic acid and 12-O-methoxy carnosic acid) were increased after 3 weeks of irrigation with 75 or 100 mM NaCl [30]. The influence of salt stress on the growth and chemical composition of the essential oils of different *Mentha* species (peppermint, pennyroyal and apple mint) has been also studied [31]. The results showed that the responses of different species to salt stress is dissimilar and only in pennyroyal, the oil concentration in the plant tissue was enhanced by salt stress [32].

Therefore, the salinity stresses can be stimulated the synthesis and accumulation of polyphenols, because these compounds participate in detoxification and plant protection against produced ROS in this condition. Thus, salt-stressed plants can be used as potential sources for the production of phenolic compounds for application in economical and industrial fields [11, 12].

4. Conclusion

Our findings showed that at higher salinity levels, more amounts of diosmin were produced in shoots of hyssop plant. Total flavonoid content of salt stressed plants was also higher than of the control group. The amount of diosmin was significantly increased in salt stress condition and diosmin content more encouraged by 150 and 200 mM NaCl. Accordingly, the highest amount of diosmin was achieved after the exposure of plants to NaCl concentration of 200 mM. Therefore, salt-stressed hyssop plants can be used as potential sources for the production of diosmin and its application in the food industry.

Declarations

Author contribution statement

Masoud Modarresi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Zhaleh Soheilikhah: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ali Movafeghi: Analyzed and interpreted the data; Wrote the paper.

Naser Karimi: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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