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Morphological and physiological response of some Iranian ecotypes of *Leonurus cardiaca* L. to drought stress

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ABSTRACT

Purpose: Motherwort (*Leonurus cardiaca*), a medicinal plant of the Lamiaceae family, has a great diversity and wide distribution in Iran. It is essential due to having leonurine with blood dilution and muscle relaxation effects. **Research method:** To evaluate the response of the Iranian Motherwort ecotypes in drought environments, an experiment was carried out in split plot design based on RCBD with three replications. Three irrigation levels were considered as main plots, including %100, one-third, and two-third of field capacity, and four ecotypes as sub-plots including Kerman, Taleghan, Sarab and Khansar. The experiment was conducted at Horticultural Research Station, the University of Tehran, in Karaj. **Findings:** The results showed that water stress affected morphological traits significantly. Water stress had a significant increasing effect on the phenolic and flavonoid contents and antioxidant activity of Motherwort ecotypes. The highest and the lowest total phenol content were observed in Taleghan and Kerman ecotypes, 7.34 and 4.21 mg/mg fresh weight, respectively. The amount and the percentage of leonurine were increased by water stress. The highest and the lowest values of leonurine were observed in the ecotypes of Taleghan and Sarab, respectively, in all three levels of irrigation. There were significant correlations between dry weight (-0.43), antioxidant (0.36), proline (-0.35), catalase (0.4), and the percentage of leonurine. **Research limitations:** There were no limitations to the report. **Originality/Value:** It was found a significant variation among the ecotypes in response to water stress. The ecotypes of Taleghan and Sarab were the most tolerant and the most susceptible ecotypes to drought stress among the studied ecotypes.

INTRODUCTION

The amount of rainfall in many parts of Iran does not provide a minimum supply of water for plant growth. Dealing with water stress, especially in summer, is inevitable for plants. Therefore, irrigation is necessary to compensate water deficit and obtain adequate yield. Replacing the plants with high drought tolerant ones is a significant aim in front of drought in arid areas. Species and ecotypes of a plant do not have the same reaction to water stress in the same condition. Obviously, ecotypes, which are more tolerant to drought stress, are more appropriate to select in arid areas (Upadhyaya & Panda, 2004). Numerous research papers have been published on the effect of drought stress on crops; however, the behavior of medicinal and aromatic plants under drought stress conditions has been studied less.

Motherwort (*Leonurus cardiaca* L.) is one of the medicinal plants that can be considered for arid areas. This plant is a perennial plant that belongs to the Lamiaceae family, has a wide distribution in Iran (Mozaffarian, 2006). Based on the researches, chemical constituents of this plant include flavonoids such as genkwanin, isoquercitrin, epigenine; triterpenoids such as ursolic acid, and corosolic acid and alkaloids such as stachydrine, leonurine, and betonicine. The products made from the plant extract are widely used to treat PMS (premenstrual syndrome), high blood pressure, stress, labor pain, heart problems, and palpitations. The extract can strengthen the nervous system, reduce spasms, increase blood circulation, and reduce thyroid hormone production. However, this plant is not cultivated in Iran but can be considered as a plant for plant domestication programs in water-limited part of Iran.

The investigation of different literatures showed no information about the impact of water stress on morphological, physiological, and photochemical parameters of *Leonurus cardiaca*; however, there is a limited study on the other species of *Leonurus*. Huanyong et al. (2013) investigated the effect of water stress on *L. japonicus* and found that the biomass increased during the stress period, but in control plants were more than others. Studies on different plants showed an inverse relationship between the increasing severity of the drought, and the number of leaves formed in *Triticum aestivum* (Abdalla & El-Khoshiban, 2007). They stated that by increasing the severity of the drought, the shoot length of treated plants was correspondingly declined beneath the control ones. Increasing duration and severity of stress decreased the leaf area, especially in the sustainable plants demonstrated by Choi et al., (2000), Garg et al. (2001), Koyro (2006), Martin and Stephens (2006), Abdalla and El-Khoshiban (2007). The fresh weight was diminished by the severity of stress, consistent with Misra and Srivastava (2000), and Mayak et al. (2004). On the other hand, Kobashi et al. (2000) found that the level of drought controls the fresh weight of peach, such the later increases with moderate drought but decreases with severe drought in peaches.

Hence, the aims of this study are evaluation of the effect of drought stress on morphological, physiological, and phytochemical characteristics of Motherwort, and identification of tolerant and susceptible Motherwort ecotypes.

MATERIALS AND METHODS

Plant material

Seeds of four ecotypes of Motherwort collected from four different regions of Iran, including Kerman, Taleghan, Khansar, and Sarab, were used in this study. The seeds were pre-treated by cooling at 4°C for 8-10 weeks. Then, they were planted in culture trays containing coco-peat and perlite with the same ratio. The growing plants were transplanted to the field at 6 to 8 leaf stage in early spring in the first year. Plots were made in 3×3 m^2. Seedlings were planted at 30 and 70 cm within and between rows, respectively, to reach a density of 4.8 plants per square meter (44 plants in each plot).

The experiment was conducted in a split-plot design based on randomized complete block design (RCBD) with three replications. Different levels of irrigation were considered as the main factor and four ecotypes as the sub-factors. The evaluations were made for two consecutive cropping seasons of 2012, and 2013 at Horticultural Research Station, the University of Tehran in Karaj (35° 39' N, 51° 06' E, and altitude of 1271 m). The maximum and minimum temperatures were 36.8 and 2°C, respectively. The mean moisture regime was 38 percent and the average annual rainfall was 268.4 mm.

Different levels of irrigation were up to full, two-third and one-third of field capacity (FC), as control, mild and severe stresses, respectively. A week after the plants were established, drought stress was applied by drip irrigation. For this purpose, the amount of calculated FC was equal to 28.41, and then the amount of soil moisture near the root was measured using Delta-T Theta probe Manufacturing Co. VS Instrument Pvt. Ltd. The amount of required water for irrigation (liter per plot), was equal to the area × the root depth × soil moisture deficit (the difference between units and FC) × the coefficient of wetted surface field (ratio of width wetted dropper and the row distance) × 10. Then 1/3 and 2/3 were multiplied by the result for severe, and mild treatments. The same agricultural practices were used for all plants in all treatments. Phenotypic assays were done in both growing seasons. Some of them, such as plant height, leaf area, and length of inflorescence, were evaluated during a full flowering stage at the end of spring. The whole plants were harvested at the flowering stage, and fresh and dry shoot weights (biomass) were measured.

Physiological and phytochemical assays were done only in the second growing year. For physiological analysis, during the blooming state, developed fresh leaves were collected from each labeled plant, frozen in liquid nitrogen, and kept in -80 C for further analysis. Proline content was estimated according to the method adopted by Bates et al. (1973). The amount of protein was determined using the spectrophotometric method recommended by Giannopolitis & Ries (1977). Enzyme activities of catalase, superoxide dismutase, and guaiacol peroxidase were determined using the methods of Packer (1984), Dehindsa et al. (1981), Maehly and Chance (1954), respectively. Methanol extraction of dry material was prepared for measuring total phenol, flavonoid, and antioxidant characteristics. The method of extraction and clarification of total phenol was similar to those described by McDonald et al. (2001). Flavonoid content was conducted by the colorimetric method of aluminum chloride described by Chang et al. (2002). The antioxidant activity of the extracts was evaluated by the method of radical scavenging of DPPH recommended by Sun et al. (2007).

Leonurine was determined by the modified method of Zhu et al. (2014) after extraction by Zhi et al. (2004) method; 2g of the dry raw plant material were loaded into the soxhlet apparatus and refluxed with ethyl acetate till the refluxtant become colorless. The ethyl acetate was evaporated by the rotary evaporator. Afterward, 25 ml ethanol and several drops of HCL (0.1 mol/l) were added to extract and treated with ultrasonic for 30 min and then filtrated. The filtrate was concentrated and shifted into 10 ml volumetric flask. The residue powder and filter paper were washed several times with ethanol. The final volume of the solution reached to 10 ml by adding ethanol. Leonurine was separated and quantified by HPLC (Knauer Co., Germany).

HPLC was optimized for all samples after investigating several conditions. The separation was performed using a Eurospher 100-5-C18 column. The mobile phase consisted of acetonitrile (solvent A) and ammonium acetate buffer (10mM, pH 4.0) (solvent B). The analysts were eluted using the following gradient program: initial composition was 10% solvent B followed by a linear gradient within 5 min, then 50% solvent B for 30 min, finally 100% solvent A for 10 min, maintained at 282 nm wavelength at a flow rate of 0.25 ml/min.

Data analysis

The variance analysis of phenotypic data and the means comparisons based on the LSD test, P ≤ 5% were analyzed by SAS ver. 9, and the correlation among traits was made by SPSS 19 software.

RESULTS

Morphological assays

The results indicated a significant influence of drought stress on motherwort phenotypic traits in two years. The severe condition affected the shoot dry weight of *L. cardiaca* in the first growing year, but the mild condition did not change this trait (Table 1). In the second growing year, when plants were slightly adapted to new environmental conditions, applied water treatments did not influence shoot dry weight. Changes in the number of inflorescence in both years showed the same trend; severe stress caused the reduction, while mild stress had no considerable effect in this trait. Reduction in the amount of dry matter due to stress has been reported by Khorshidi et al. (2019), Abdalla and El-Khoshiban (2007), and Monti et al. (2006) in *Beta vulgaris*. Abdalla and El-Khoshiban (2007) showed a reduction in dry weight under water stress due to the stimulation and regulation of photosynthetic enzymes. In addition, Babaee et al. (2010) reported the reduction in the number of lateral branches in thyme.

Morphological characteristics of the ecotypes

During the first growing year, the ecotype of Kerman had the lowest dry weight, and a significant difference was not observed among the other ecotypes, while in the second growing year, the lowest dry weight was found in the ecotype of Sarab, and there was no significant difference among the others (Table 2).

Table 1. Means of the morphological traits measured in different ecotypes of motherwort under different levels of water stress in two growing years (2012-2013)

Water stress	Shoot Dry Weight (g) (2012)	Number of inflorescence		Interval length of two floral cycles (cm) (2013)	Number of lateral branches (2013)
		2012	2013		
Control*	13.79 a	38.48 a	50.36 a	2.64 b	41.57 a
Mild	10.47 ab	28.47 a	40.47 a	2.97 ab	29.79 b
Severe	8.46 b	17.45 b	29.38 b	3.36 a	2.16 b

The means with at least one same letter are not significantly different at 5% probability level.
*Control: 100% of field capacity (FC), mild: two third of FC, severe: one third of FC

Table 2. Means of the motherwort ecotypes for the morphological traits in two years (2012-2013)

Trait	Year	Ecotype			
		Kerman	Taleghan	Sarab	Khansar
Dry Weight (g)	2012	9.25 b	11.34 a	12.03 a	10.99 a
	2013	76.45 a	79.22 a	59.18 b	74.21 a
Number of lateral branches	2013	36.43 a	29.84 ab	33.61 a	22.15 b

The means in each row with at least one same letter are not significantly different at the P≤5%

Means of the ecotypes over drought stress levels for plant height are shown in Figures 1 and 2. The result of this study showed that the first growing year and in all ecotypes except Taleghan, an increase in water limitation caused a decrease in plant height indicated that the Taleghan ecotype tolerates drought effects better than others.

The decrease in plant height under water stress was reported by Babaee et al. (2010) and Eman et al., (2008) in *Thymus vulgaris*, Alkire et al., (1993) in *Mentha piperita*, Baghalian et al., (2011) in *Matricaria recutita*. The water deficit decreases cell turgor follows by a decrease in cell growth and development, which causing a limitation in plant growth, and its first effect is a decrease in plant height (Babaee et al., 2010). In a survey conducted by Soorni (2013) on the wild plants of the ecotypes used in this research, the highest plant height belonged to Taleghan as seen in control plants in the second growing year of present study and Kerman.

Physiological assays

No significant difference in the concentration of proline was observed in the ecotypes of Taleghan and Sarab (Fig. 3). However, in Kerman there was an increase in proline concentration of severe water shortage with respect to both well and mild-watered conditions. Khansar showed an increase in proline concentration in mild and severe conditions but this increase was lower in severe compare to mild condition. An increase in the value of proline in drought stress conditions is the result of a decline in the osmotic potential of the cell. Inotai (2013), by studying three levels of stress (70, 50 and 30% of field capacity) on savory (*Satureja hortensis* L.), and Basil (*Ocimum basilicum*) showed that by reducing soil water content, proline content was increased in both plants. These results also agreed with the findings of Zhang et al. (2006) in Seville, and Abdalla & Alkhoshiban (2007) in wheat (*Triticum aestivum*).

The well-watered plants of both ecotypes of Kerman and Khansar showed the highest values of protein concentration (Fig. 4). No significant decrease was observed in the Kerman ecotype under water stress compared the control condition. However, in the other ecotypes occurred a significant decline in protein concentration under water stress. The results were adapted with Setayeshmehr & Ganjali (2013), Mohammadkhani & Heidari (2008). According to Mohammadkhani and Heidari (2008), a decrease in the soluble protein of corn treated with drought stress is because of a sharp drop in photosynthesis and subsequent reduction in the production of protein precursors and, ultimately decrease in protein synthesis. Some researchers have linked depression of protein synthesis to decline in the number of polysomes binding to the membrane (Mohammadkhani & Heidari, 2008). Feller et al. (2004) reported that drought stress promotes the expression of genes inducing intracellular proteases and causes protein degradation and remobilization of nitrogen and subsequent synthesis of compatible soluble compounds.

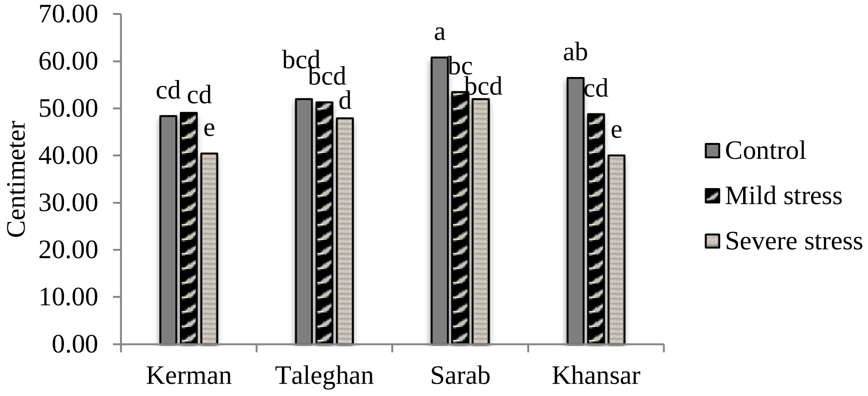

Fig. 1. Means of plant height in the motherwort ecotypes under water stress levels in the first growing year (2012).

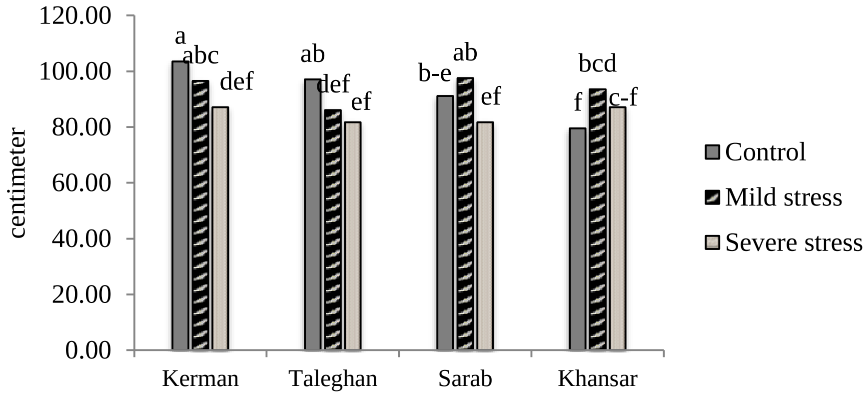

Fig 2. Means of plant height in the motherwort ecotypes under water stress levels in the second growing year (2013).

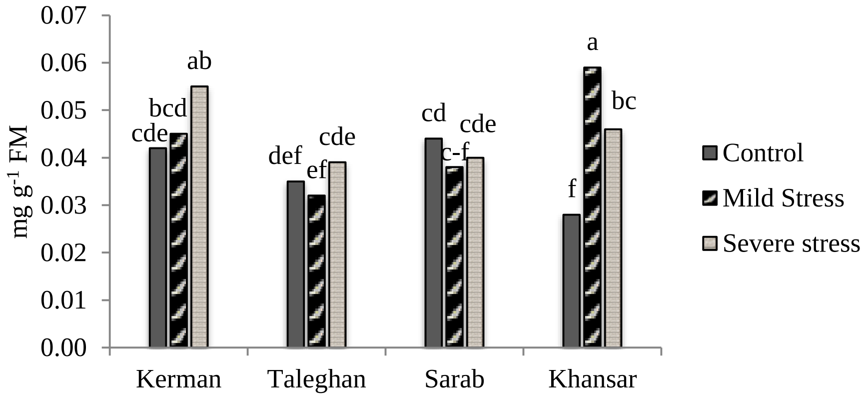

Fig 3. Means of proline in some Motherwort ecotypes under water stress levels.

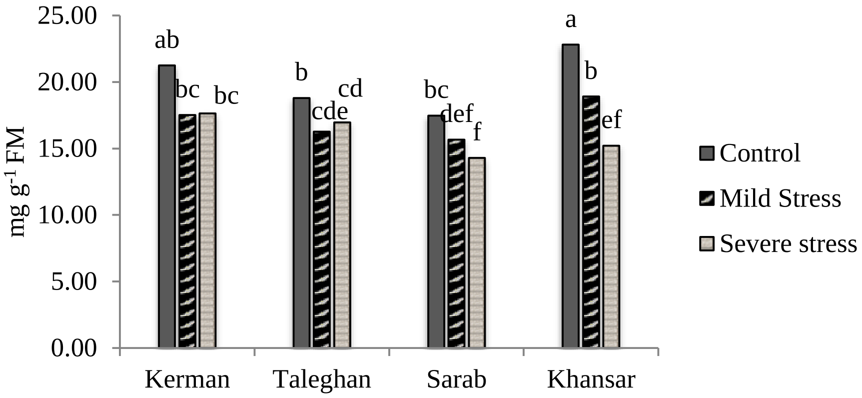

Fig. 4. Means of protein in some motherwort ecotypes under water stress levels.

Biochemical and genetic studies confirm that hydrogen peroxide is a signaling molecule in plants that mediates responses to abiotic and biotic stresses (Neill et al., 2002). The greatest and the least amounts of hydrogen peroxide were observed in the ecotypes of Sarab and Taleghan in non-stress condition, respectively (Fig. 5). The increased concentration of hydrogen peroxide under water stress in Kerman and Khansar was not significant. However, in Taleghan and Sarab, water stress caused a significant increase concerning well-watered condition. Al-Ghamdi (2009) also reported an increase of hydrogen peroxide as toxic metabolites under drought stress in wheat. Hydrogen peroxide is produced in the majority of cellular organelles under critical metabolic processes. Reduce or stop the activity of these enzymes finally decreases the absorption and fixation of carbon dioxide (Vaidyanathan et al., 2003). It should be noted that high levels of hydrogen peroxide make oxidation of the thiol groups that the outcome can be disabled or unwanted activation of key enzymes (Neill et al., 2002).

In general, the control plants of the Kerman and Taleghan ecotypes, and all the drought treatments of Sarab had the lowest amounts of catalase activity with no significant differences. The drought-stressed plants (mild, and severe conditions) of Kerman, and severe-stressed plants of Khansar ecotypes had the highest amount of catalase activity (Fig. 6). Catalase is an oxidoreductase, located in peroxisomes and considered as an essential enzyme to measure hydrogen peroxide in stress condition. The activity of this enzyme is sensitive to water stress.

Higher activity of catalase (CAT) decreases H_2O_2 level in the cell (by breaking down the H_2O_2 to water and oxygen). It increases the stability of membranes and CO_2 fixations because of several enzymes of the Calvin cycle within chloroplasts are susceptible to H_2O_2. A high level of H_2O_2 directly inhibits CO_2 fixation (Yamazaki et al., 2003).

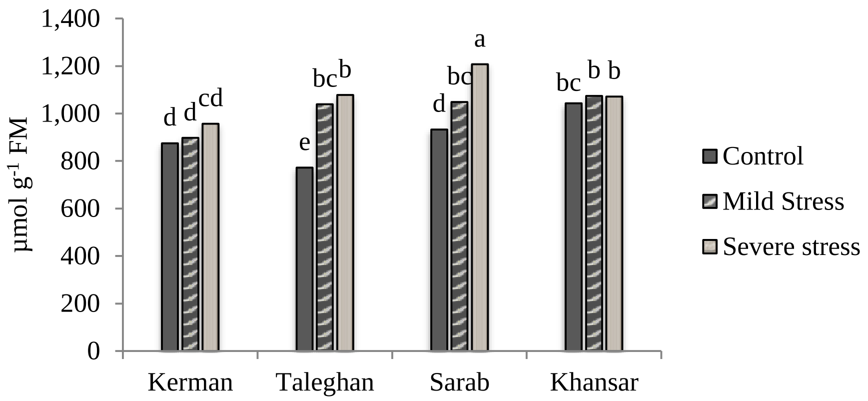

Fig. 5. Means of hydrogen peroxide in some Motherwort ecotypes under water stress levels

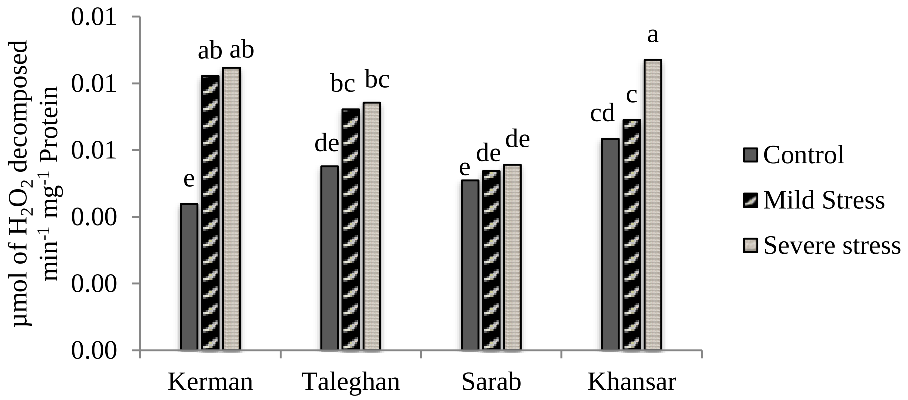

Fig. 6. Means of catalase enzyme activity in some Motherwort ecotypes under water stress levels

DISCUSSION

The results of this study showed that an increase in the magnitude of water stress caused the increase in the SOD activity of Khansar and Kerman ecotypes (Fig. 7). The highest and lowest superoxide dismutase was registered in Kerman and Sarab under severe drought stress, respectively. Increasing superoxide dismutase under drought stress has been reported by Al-Ghamdi (2009) in wheat and Yang et al. (2006) in *Radix astragali*.

The changes in the enzyme activity of guaiacol peroxidase showed a similar trend to SOD in the studied ecotypes (Fig. 8). Enayati (2010) reported a rise of guaiacol peroxidase enzyme activity in some wheat varieties under salt stress. Guaiacol peroxidase enzyme converts toxic metabolites of hydrogen peroxide into water. Hence, the increase of activity of this enzyme during stress causes the collection and removal of hydrogen peroxide. Cooperation among the enzymes to collect reactive oxygen species is essential. Therefore, in scavenging of reactive oxygen species, participation and cooperation of several antioxidant enzymes are needed in this regard.

Table 3. Means of antioxidant activity, total phenol and total flavonoids in different water stress conditions

Treatment	Antioxidant activity	Total phenol (mg g^{-1})	Total flavonoids (%)
Control*	92.76 b	4.31 b	6.21 b
Mild stress	102.70 b	4.74 b	7.29 b
Severe stress	116.16 a	8.72 a	9.47 a

*Control, mild and severe are full, two third and one third of field capacity, respectively.

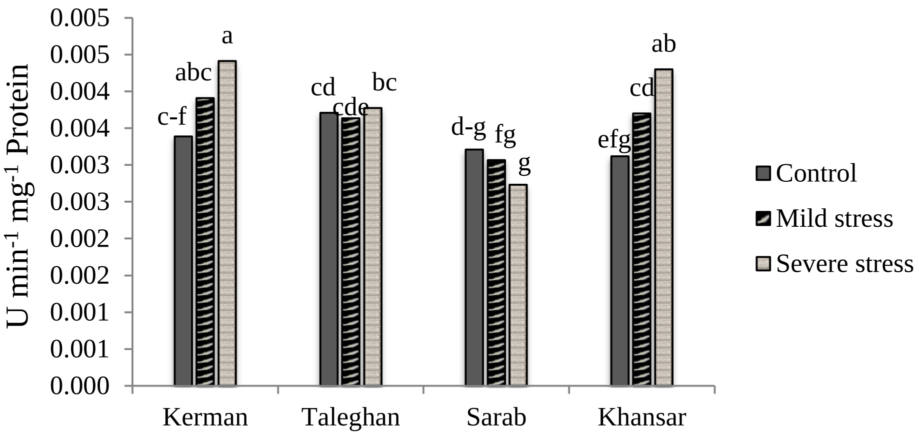

Fig. 7. Means of superoxide dismutase in some Motherwort ecotypes under water stress levels

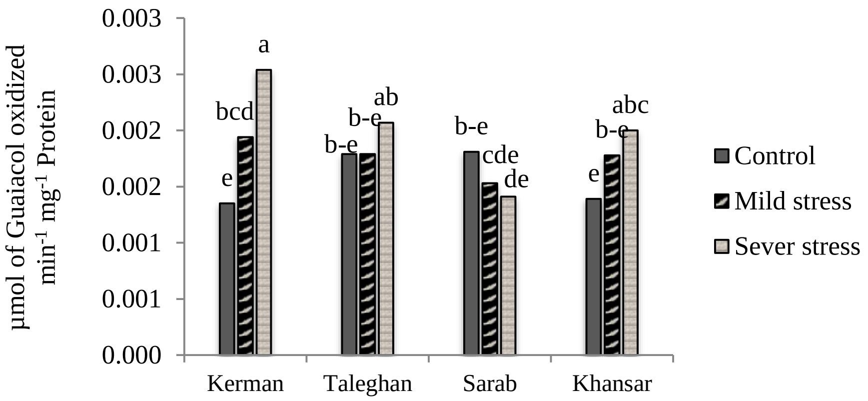

Fig. 8. Means of guaiacol peroxidase enzyme activity in some Motherwort ecotypes under water stress conditions

According to the results (Table 3), there was a significant difference in different stress levels regarding the amount of anti-oxidant, total phenol, and total flavonoids. Therefore, severe stress significantly increased the amount of every three traits, while no significant difference was observed between control and mild stress. An increase in phenolic compounds under stress was consistent with the findings of Setayeshmehr and Ganjalikhani (2013), Latha et al. (2007). The increased total flavonoid content under stress was compatible with the findings of Huanyong et al. (2013).

The consequence of antioxidant mechanisms of plants under drought stress is the increase in the quantity of phenolic compounds, because these compounds act as the purificatory of reactive oxygen species, resulting in the stabilization of cell membranes and inhibit lipid peroxidation (Chang et al., 2002).

Investigation of total phenol in the ecotypes showed that Taleghan and Kerman had the highest and the lowest amount, respectively. Still, no significant difference was observed between Kerman and two other ecotypes (Fig. 9). Soorni et al. (2013) demonstrated that the amount of total phenol ranged from 4.6 to 23.2 gr Galic acid g^{-1} in some ecotypes of *L. cardiaca*. The result of this study was in agreement with findings of Mantle et al., 2000, Bernatoniene et al., 2009, and Armatu et al., 2010. Also, Abdi et al (2019) reported an increase in phenol content due to water stress in *Mentha piperita.*

The mean comparison of water stress levels on leonurine percentage showed that increasing the level of water stress led to elevate leonurine percentage (Table 4, Fig. 10). So that the minimum and maximum amounts and percentages of leonurine were obtained in control and mild stress conditions, respectively. Based on the result, the presence of leonurine was confirmed in specimens of *L. cardiaca* from Iran as previously reported (Wichtl, 2009; Kartnig et al. 1993; Bradley, 1993; Schantz, 2009; Hiller & Melzig, 2007; Barnes et al. 2007; Shikov et al., 2011).

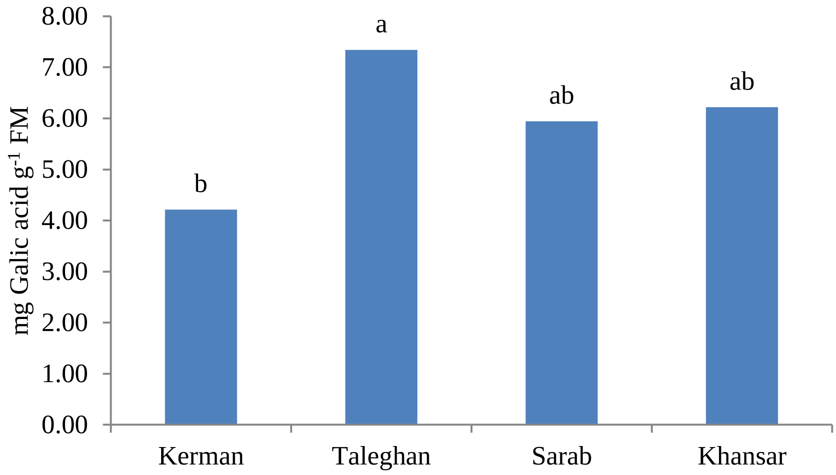

Fig. 9. Means of Motherwort ecotypes for total phenol

Table 4. Means of leonurine in different ecotypes of Motherwort under different treatments of water stress

Stress	Leonurine (%)	Leonurine (mg/2g sample)
Control	0.071 [c]	1.47 [b]
Mild Stress	0.091 [b]	1.83 [ab]
Severe Stress	0.11 [a]	2.20 [a]

The same letters in each column indicate the lack of significant differences among the means.

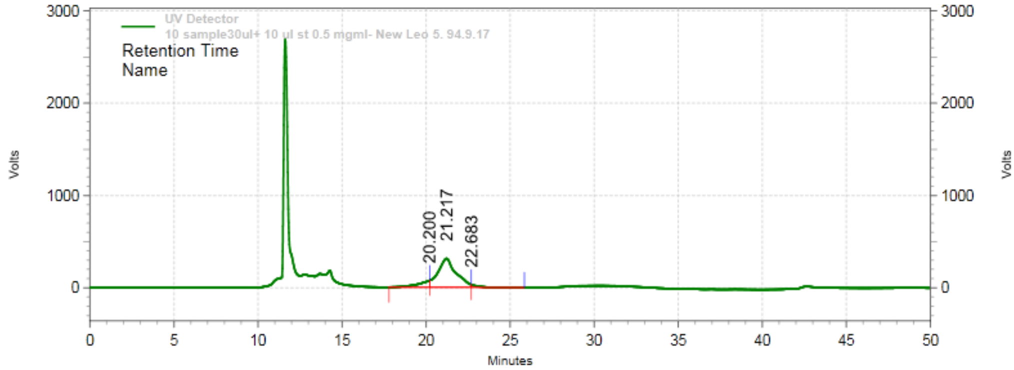

Fig. 10. Chromatogram of leonurine in Motherwort under the control treatment using HPLC

CONCLUSION

Using abiotic stress is one of the approaches to change the components and the amount of active ingredient of medicinal plants that many studies have been done in this case. The results of this study showed that water stress had significant effects on morphological traits, dry weight, active substance content, and physiological traits of *Leonurus cardiaca*. Some ecotypes, such as Khansar showed more stability in morphological characteristics in both first and second growing years.

An increase in water stress levels reduced the amount of dry weight and number of inflorescences in the first growing year and the number of inflorescence and branches in the second growing year.

Proline content increased in Khansar, and Kerman under the water stress, while Protein decreased in all ecotypes except Kerman. An increase in the stress intensity caused the growth in enzyme activities. Since co-enzymes in the collection of reactive oxygen species, especially hydrogen peroxide, are essential, and high activity of these enzymes is one of the drought tolerance mechanisms; therefore, Sarab ecotype regarding of the low activity of superoxide dismutase and catalase enzymes under the stress, and the subsequent accumulation of hydrogen peroxide is more susceptible to drought than the other ecotypes.

Generally, a decrease in protein content was observed in different ecotypes and the greatest content was found in Khansar under the control treatment. The effect of stress on the content of total phenol, flavonoid, and antioxidant of Motherwort ecotypes demonstrated an increase in the value of these traits during different levels of drought stresses. The highest and lowest total phenol content was observed in Taleghan and Sarab, respectively.

The results indicated that leonurine occurred in different drought-stressed ecotypes of *L. cardiaca*. The result of present study is inconsistent with the findings of Kuchta et al. (2012). The amount and percentage of leonurine affected by drought and increased so that the maximum amount of leonurine was observed in the treatment of mild stress and the lowest one in control plants. However, the ecotypes were not significantly different in terms of the alkaloid of leonurine. Leonurine yield was the most in the Taleghan ecotype, while the lowest leonurine was seen in ecotype of Sarab on all three levels of control, mild and severe stresses.

An increase in dry weight represents the optimum conditions for plant growth in favor of relevant biosynthetic pathways and production of primary metabolites, which in consequence, led to a reduction in the secondary metabolite production.

Antioxidant activity had a significant positive relationship with leonurine, proline, guaiacol peroxidase, and superoxide dismutase. The increase in later enzymes, and proline and leonurine is a way to increase the plant defense system against abiotic stresses, in particular oxidative stress that enhances the antioxidant effects of plants.

Generally, according to morphological, physiological, and phytochemical results, it could be demonstrated that the motherwort is capable of adapting to dry weather. However, more breeding studies need to introduce it to cultivation in arid regions. Among studied ecotypes, Sarab showed a low tolerance to water stress in the most traits, so, it can be introduced as a susceptible ecotype. In contrast, Taleghan and Khansar showed a high tolerance to water stress and can be presented as a tolerant ecotype.

Conflict of interest

The authors have no conflict of interest.

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