Effect of ethanol and humic acid foliar spraying on morphological traits, photosynthetic pigments and quality and quantity of essential oil content of *Dracocephalum moldavica* L.

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Abstract

In order to study the effects of humic acid foliar spraying on morphological traits, photosynthetic pigmentation, and essential oil content of *Dracocephalum moldavica*, a factorial experiment was conducted based on a randomized complete block design with three replications in Saveh. The investigated factors included the use of humic acid at four levels 0 (control), 200, 400, and 800 mg/L and ethanol at four levels of 0 (control), 5, 10, and 15%. The measured traits were biological yield, chlorophyll a, chlorophyll b, total chlorophyll, carotenoid, essential oil percentage, and essential oil yield. Results showed that humic acid increased biological yield, chlorophyll a, chlorophyll b and total chlorophyll, essential oil percentage, essential oil yield, carotenoid, and free sugar. The highest positive effect was observed in 400 mg/L humic treatment. Ethanol increased biological yield, chlorophyll a, chlorophyll b and total chlorophyll, essential oil content, essential oil yield, carotenoid, and free sugar. In general, the highest positive effect was obtained in 10% ethanol treatment. Results showed that the use of humic acid with ethanol, especially in the combination of 400 mg/L humic acid with 10% ethanol, improved morphological characteristics, photosynthetic pigments, and yield of essential oil of *Dracocephalum moldavica.*

Keywords: ethanol; humic acid; chlorophyll; ssential oil content; *Dracocephalum moldavica*

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Introduction

Dracocephalum moldavica L. is an annual herbaceous aromatic plant belonging to Lamiaceae family which is native to Central Asia and is naturalized in eastern and Central Europe (Dastmalchi et al., 2007). All organs of the plant contain essential oil with varying contents in different parts which is used widely in food, flavoring, pharmaceutical, and cosmetic industries. Vegetable flowers and lichens (young leaves and stems) have the highest percentage of essential oil which also show anti-tumor properties (Hussein et al., 2006). Essential oil of *Dracocephalum moldavica* is a bright, light yellow liquid with a very pleasant, very penetrating and

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Table 1
Some physical and chemical properties of soil test in the field experiment

Sand (%)	Silt (%)	Clay (%)	pH	$EC*10^6$ (dS/m)	Total Nitrogen (%)	Organic carbon (%)	P (ppm)	K (ppm)	Ca+Mg (cmol/kg)
37	42	21	8	480	0.16	0.21	8.43	187	42.51

tasty aroma, which has anti-bacterial and antioxidant properties and is used for the treatment of abdominal pain and abdominal bloating (Omidbaigi, 2005). The main components of the essential oil of this plant are Geranial, Naral, Geranil acetate, and Geranium, which are ring-shaped monocrystalline oxygenic and make up 90% of essential oils (Omidbaigi et al., 2009).

Humic matter is formed through the chemical and biological decomposition of plant and animal matter and through the activities of microorganisms (Metzger, 2010). Humic acid is one of the most suitable fertilizers used in the organic farming system. Regarding environmental considerations, organic acids have been used to improve the quality and quantity of crops and gardens. Application of humic acid to foliage and to soil increases auxin, cytokinin, and gibberellin levels in plants (Abdel Mawgoud et al., 2007). The consumption of humic acid increases the dry weight of the shoot, height, number of flowers, and leaves in the flower of spring flowers (Mohammadipour et al., 2012). Due to the positive effects on nutrient uptake, humic acids increase growth and yields of some vegetables (Zandonadi et al., 2007; Cimrin and Yilmaz, 2005). Foliar application of humic acid and on Asparagus plants increase carbohydrates production, chlorophyll, and carotenoids in edible stems and uptake of some elements in shoot (Cangi et al. 2006). Total chlorophyll content significantly increased in response to both foliar and soil humic acid treatments (Karakurt et al., 2009). Moreover, studies explaining the effects of humic acid suggested that humic acids demonstrate their effects through increasing enzyme catalysis, enhancing respiration and photosynthesis, and stimulating nucleic acid metabolism (Serenellaet al., 2002). The method of spraying with alcohol, in particular ethanol, is one of the effective and appropriate ways to increase the production in agricultural products, especially medicinal plants. Alcoholic treatments can increase the accumulation of carbohydrates, increase the concentration of carbon dioxide, and accelerate flowering. Increasing the concentration of carbon dioxide can neutralize the effects of environmental stresses (Zbiec et al., 1999),

Dracocephalum moldavica essence has anti-bacterial properties and is used to treat bloating and abdominal bloating. Essential oils are also used in the food, beverage, health, and beauty industries (Dmitruk and Weryszko-Chmielewska, 2010). Application of organic manure can pave the way to replenishing the essential nutrients and improving crop productivity (Bajeli et al., 2016). The aims of the present work were to study the effects of ethanol and humic acid spraying on morphological traits, photosynthetic pigments, and essential oil contents of *Dracocephalum moldavica*.

Materials and Methods

This experiment was carried out as a factorial based on randomized complete block design with three replications in 2015-2016 in an experimental field in Saveh (latitude: 35°, 0´ N; longitude: 50°, 22´ E; altitude: 978 m above sea level). Land preparation including plowing, disking, rotavating, and classification of farm plots were done in winter 2015. Some physical and chemical properties of soil used in the study were determined according to Jackson (1973) as presented in Tables 1. The experimental plots' size was 2.5 × 2.5 m. Each plot consisted of 6 rows of planting with a distance of 40 cm. Seeds were obtained from Seed and Plant Improvement Institute of Iran. The obtained seeds were disinfected with 5% sodium hypochlorite for 5 minutes and distilled 3 times with distilled water. In May 2015, the seeds were first sliced and then 3-5 seeded were sown 5 to 1.5 cm deep which were subsequently thinned in the third week.

The investigated factors included the use of humic acid at four levels of non-consumption (control), 200, 400 and 800 mg/L and ethanol at 0, 5, 10 and 15% were applied as a foliar spray. Plants were irrigated twice a week after planting and weeding was carried out manually. Treatments were sprayed on the plants when they were completely established. Ethanol was sprayed on the plants at three stages of the plant development and after two days the plants were leached and then sprayed with the humic acid solution. Foliar application was carried out at three stages of growth, namely, four-leaf stage, before flowering, and at flowering stage. At the end of flowering ten plants per plot were selected randomly and the traits were measured. For dry weight measurement, the bushes were harvested and put in an oven set at 72 °C for 48 hours. In order to determine the biological functions, the harvest was carried out at a surface area of 2.25 square meters after removing the marginal effects in each plot from two lateral rows and 0.5 m from the beginning and the end of the rows of planting.

For chlorophyll measurement, 0.2 g of leaf samples was extracted using 80% acetone. The extract was then placed on a filter paper and reached 25 ml. Absorptions of chlorophyll a and b were read at 645 and 663 nm, respectively, and the concentrations of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid were calculated using the following equations (Arnon, 1949):

Chlorophyll a (mg/g fresh tissue) = 12.7 $A_{(663)}$- 2 .69 $A_{(645)}$ × V/(1000 × W)

Chlorophyll b (mg/g fresh tissue) = 22.9 $A_{(645)}$- 4.68 $A_{(663)}$ × V/(1000 × W)

Total Chlorophyll (mg/mL) = Chlorophyll a + Chlorophyll b.

Carotenoid (mg/g fresh tissue) = $A_{(475)}$10 × V/(2500 × W)

A645 = absorbance at a wavelength of 645 nm

A663 = absorbance at a wavelength of 663 nm

A475 = absorbance at a wavelength of 475 nm
V: Final volume of the extract

W: Fresh weight of leaves taken for extraction

Essential oils from shoots were extracted with water using steam distillation method. The essential oil was extracted from samples from by distillation with water for 4 h. Extracted essential oil was dehumidified by dry sodium sulfate and then the percentage of essential oil was determined. To analyze essential oil and accurately measure its ingredients, gas chromatography was used. GC–MS analysis was performed using a Hewlett Packard 5890A apparatus coupled to a VG-TRIO-2 quadrupole mass spectrometer with a direct capillary column with 30 m length, 0.25 mm internal diameter and 25 mm thickness (Agilent/J and W Scientific, Folsom, Ca, USA). The initial oven temperature was set at 80 ˚C for 2 minutes and then was ramped up at 10 ˚C per minutes to 140 ˚C held for 1 min after which it was ramped up at 4 ˚C per minute to 190˚C and held for 2 minutes and again it was ramped up at 2 ˚C per minutes to 210˚C. The sample (1 ml) was injected with a split ratio of 1: 10. The carrier gas was helium at flow rate of 1.0 ml for 1 min. Output peaks based on retention times were compared with standard samples and the concentration was determined based on the area under the curve (Adams, 2007). Relative percentages of the components were determined using normalization method.

Soluble sugar was determined from fresh leaves at the flowering stage using Arnon method (1949). Proline accumulation was carried out by extracting fresh samples in 3% sulfosalicylic acid using Troll and Lindsley method (1955). Data analysis was performed using SPSS statistical software and means were compared using Duncan test at $p \leq 0.05$.

Results

Biological function

Results of analysis of variance indicated a significant effect of humic acid and ethanol ($p \leq 0.01$). Also showed the interaction of humic acid with ethanol on biological yield was significant at 5% probability level (Table 2). Comparison of means showed that the average biological yield under the influence of humic acid factor along with the application of humic acid increased biological yield, so that the highest biological yield was 1873.5 kg/ha in the treatment with 400 mg/L humic acid and the lowest biological yield (1430.08 kg/ha) was obtained in the control treatment (Table 3). Moreover, ethanol increased biologic performance compared with the control and the highest biological yield was 1787.48 kg/ha in the 10% ethanol treatment. The average biologic performance was influenced by the interaction between humic acid and ethanol (Table 3) and the highest biologic yield (2157.07 kg ha-1) was recorded in the treatment of 400 mg/l humic acid in the presence of 10% ethanol.

Chlorophyll a

The exogenous application of ethanol (0, 5, 10, and 20%) and humic acid on Chlorophyll had significant effects at 1% probability level (Table 2). According to the results, the interaction of humic acid with ethanol had a significant effect on the chlorophyll content (p≤0.05). Comparison of the mean analysis showed that the highest chlorophyll a was 0.9 mg/g fresh weight for the treatment containing 200 mg/L humic acid (Table 3). Moreover, the highest chlorophyll a content (0.91 mg/g) fresh weight was related to 5% ethanol treatment while the lowest chlorophyll a content was 0.7 mg.

Chlorophyll b

Data presented in Table 3 illustrate that humic acid, ethanol and the interaction of humic acid with ethanol at 1% probability level have significantly effects on chlorophyll b. The results of the comparison of the mean of chlorophyll b under the influence of the humic acid factor (Table 4) showed that in the presence of humic acid, chlorophyll b increased compared to the control; the highest chlorophyll b value was 0.97 mg/g fresh weight in treatment 400 mg/L humic acid. The results showed that ethanol foliar spraying caused an increase in chlorophyll b and the highest chlorophyll b content of 0.98 mg/g fresh weight was obtained in 5% ethanol treatment and highest chlorophyll b value of 1.36 mg/g fresh weight was related to 5% ethanol treatment in the presence of 400 mg/l humic acid.

Table 2
Results of analysis of variance of ethanol and humic acid spraying on yield and morphological traits of *Dracocephalum moldavica*

Source of Variation	df	Biological yield	Essential oil %	Essential oil yield	Chlorophyll a
Block	2	7784.69^{ns}	0.003*	1.19*	0.01^{ns}
Humic Acid (a)	3	402354.88**	0.002**	14.48**	0.15**
Ethanol (b)	3	126624.07**	0.005**	5.28**	0.09**
a*b	9	52690.45*	0.002*	1.71**	0.03*
Error	30	23809.23*	0.001	0.36	0.01
CV%		22.9	11.43	12.73	12.52

ns: non-significant, *: significant at 0.05 level, **: significant at 0.01 level

Table 3
Results of analysis of variance of ethanol and humic acid spraying on yield and morphological traits of *Dracocephalum moldavica*

Source of Variation	df	Chlorophyll b	Total Chlorophyll	Cartonoeid	Proline	Free Sugar
Block	2	0.05^{ns}	0.11*	0.02ns	0.003ns	0.01ns
Humic Acid (a)	3	0.09**	0.43**	0.26*	0.27**	4.67**
Ethanol (b)	3	0.13**	0.41**	0.012ns	0.16*	1.03*
a*b	9	0.06**	0.14**	0.17*	0.12**	2.08**
Error	30	0.02	0.03	0.004	0.003	0.06
CV%		14.06	10.2	8.54	10.43	9.82

ns: non-significant, *: significant at 0.05 level, **: significant at 0.01 level

Total chlorophyll

Results of analysis of variance indicated that the effect of humic acid, ethanol, and the interaction of humic acid with ethanol on total chlorophyll was significant at $p \leq 0.05$ (Table 3). The comparison of the mean total chlorophyll content under the influence of the humic acid (Table 3) indicated that in the presence of humic acid, the total chlorophyll content increased to 1.821 and 1.81 mg /g wet weight was obtained in the treatments containing 200 and 400 mg/L humic acid and the lowest total chlorophyll content was 42.1 mg/g fresh weight recorded in the control. Comparison of the total chlorophyll content under the influence of ethanol (Table 5) showed that ethanol consumption increased the total chlorophyll content compared to the control, the maximum total chlorophyll content (1.55 mg/g) was in 5% and 10% ethanol.

Essential oil percentages

The analysis of variance showed that ethanol and humic acid had a significant effect on essential oil percentages. Also, the interaction of

Table 4
Mean comparisons of interaction effect of ethanol and humic acid on the evaluated traits of *Dracocephalum moldavica*

Humic Acid (mg/L)	Ethanol	Biological yield (kg/ha)	Essential oil (%)	Chlorophyll a (mg/g fw)	Chlorophyll b (mg/g fw)
0	0	1325.22g	0.107g	0.54f	0.68d
0	5	1394.04fg	0.234ef	0.68cde	0.82bcd
0	10	1408.49efg	0.247def	0.73 bcd	0.83bcd
0	15	1592.57cdefg	0.277ef	0.62ef	0.77cd
200	0	1536.75defg	0.25def	0.79bcd	0.76cd
200	5	1861.54bc	0.239bcde	1.09a	0.96bc
200	10	1734bcd	0.317bc	0.87bc	1.05b
200	15	1697.56bcde	0.303bcd	0.87bc	0.88bcd
400	0	1701.9bcd	0.277cde	0.63ef	0.71cd
400	5	1976.61ab	0.333ab	1.06a	1.36a
400	10	2135.07a	0.373a	0.75bcd	0.92bcd
400	15	1680.59cdef	0.277cde	0.92ab	0.88bcd
800	0	1615.44cdef	0.263cde	0.84bc	0.82bcd
800	5	1623.55cdef	0.264cde	0.81bcd	0.78cd
800	10	1872.14abc	0.263cde	0.81bcd	0.85bcd
800	15	1634.93cdef	0.28bcde	0.78bcd	0.86bcd

In each column, means with the similar letters are not significantly different at 5% level of probability using Duncan test.

Table 5
Mean comparisons of interaction effect of ethanol and humic acid on evaluated traits of *Dracocephalum moldavica*

Humic Acid (mg/L)	Ethanol %	Total Chlorophyll (mg/g fw)	Cartonoeid (mg/g fw)	Proline (mg/L fw)	Free Sugar (mg/g dw)	Essential oil Yield (kg/ha)
0	0	1.22f	0.21f	0.41bc	1.30e	2.25i
0	5	1.5def	0.24de	0.62ab	1.49bcd	3.41gh
0	10	1.55 def	0.23def	0.58bc	1.48bcd	3.44fgh
0	15	1.39ef	0.26de	0.61ab	2.16a	3.77efgh
200	0	1.55def	0.24de	0.34cd	1.32de	3.85efgh
200	5	2.05b	0.27de	0.36cd	1.64bc	5.45c
200	10	1.92bc	0.31c	0.28cd	1.43cd	5.48c
200	15	1.75bcd	0.30bc	0.47bc	1.65bc	5.11c
400	0	1.34ef	0.31bc	0.51abc	1.52bcd	4.74cd
400	5	2.43a	0.34bc	0.54ab	1.76ab	6.61b
400	10	1.67cde	0.36bc	0.53ab	1.43cd	7.98a
400	15	1.79bcd	0.41ab	0.63ab	1.76ab	4.64cde
800	0	1.66cde	0.39ab	0.63ab	1.38de	4.25defg
800	5	1.6cde	0.42ab	0.74a	1.52bcd	4.26defg
800	10	1.66cde	0.45a	0.72a	2.12a	4.96cd
800	15	1.64cde	0.48a	0.63ab	1.43cd	4.56defg

ethanol with humic acid had a significant effect on essential oil percentages. Similarly, the interaction of humic acid with ethanol resulted in a significant effect at the 5% probability level (Table 2). Treatments containing different levels of ethanol significantly increased essential oil percentages in comparison with control. The highest percentage of essential oil was 0.277% in the treatment with 400 mg/L humic acid (Table 4). Comparison of the means also showed that the percentage of essential oil increased by ethanol compared with control, and the highest percentage of essential oil (0.3%) was obtained under 10% ethanol treatment.

Essential oil yield

Results of analysis of variance indicated that ethanol and humic acid had a significant effect on essential oil yield. Also, the interaction of ethanol with humic acid had a significant effect on essential oil yield at the 1% probability level (Table 2). Different levels of ethanol had significantly increased essential oil yield in comparison with control. Results of comparison of means showed that the essential oil yield was affected by the ethanol and increased compared to the control and the highest essential oil yield was 3.77 kg/ha in 15% ethanol (Table 5). Results also showed that the highest essential oil yield was 7.98 kg/ha in the treatment containing 10% ethanol and 400 mg/L humic acid.

Carotenoid

The analysis of variance showed that humic acid had a significant effect on carotenoid while the effect of ethanol on carotenoid content was not significant. Moreover, the interaction of effects of humic acid and ethanol on carotenoid content were significant at 5% probability level (Table 3). Ethanol 15% increased carotenoid content of fresh weight (0.26 mg/g) compared to control (0.21 mg/g) (Table 5). The mean comparison results showed that the highest carotenoid content was 0.41 mg/g per fresh weight under 15% ethanol and 800 mg/l humic acid treatment.

Free sugar

Results of analyze variance showed that humic acid and ethanol had significant effects on free sugar at 1% and 5% probability level, respectively (Table 3). Ethanol 15% significantly increased free sugar content from 1.30% at control to 2.16%. Application of humic acid increased free sugar content significantly from 1.3 mg/g at control treatment to 1.52 mg/g at 400 mg/l humic acid (Table 5).

Proline

Effects of humic acid and ethanol on proline content are given in Table 3. Humic acid and ethanol had significant effects on proline at 1% and 5% probability level, respectively. Ethanol 15% increased proline content from 0.41 mg/l fresh weight in control to 0.61 mg/l fresh weight (Table 5). Also, the highest proline content was recorded in 800 mg/l humic acid (Table 5).

Discussion

Humic acid has been shown to increase the yield and biomass production in organic plants through positive physiological effects, including increasing the metabolism in the cells and also increasing the chlorophyll content of the leaves (Nardi et al., 2002). Foliar spray application of alcoholic beverage increases the amount of chlorophyll, photosynthetic capacity, and dry matter. The deposition of methanol leads to an increase in the amount of fructose 1, 6-bisphosphatase (FBPase), which is one of the important enzymes controlling the process of photosynthesis (Andreas et al., 1990). Furthermore, the use of humic acid increases Zn, Cu, and Mn absorption in plants. Zn is a catalyst in many plant enzyme systems which is involved in protein synthesis and analysis. One of the important roles of Zn is synthesizing the amino acid, tryptophan, which is a precursor for the auxin, indoleacetic acid promoting branch length growth; Cu plays a role in the activation of plant oxidases and Mn has an essential role in chlorophyll production in plants. Humic acid increases Mn absorption in plants which inturn plays an essential role in chlorophyll production in plants.

Results of the study showed that consumption of humic acid increased chlorophyll a, chlorophyll b, and total chlorophyll content. Humic acid can increase the production of chlorophyll by placing more water and nutrients on the plant (Delfine et al., 2005). Alcoholic treatments increase turgor pressure and the content of sugar and cellular inflammation in leaves, which helps the leaf to grow and increases chlorophyll content (Zbiec et al., 2003). Chlorophyll content is considered as an important quality of plants which is mainly responsible for the green color of the leaves. Total chlorophyll content significantly increased in response to foliar humic acid and ethanol treatments. Results showed that the highest essential oil yield (7.98 kg ha^{-1}) was related to 10% ethanol treatment with 400 mg/l humic acid while the lowest essential oil yield was 2.75 kg/ha observed in control. Phenological stage of plants accelerates by foliar application of ethanol which results in early maturation and lower water demand (Nonomura and Benson, 1992). Humic acid increases the activity of the enzyme by increasing the activity of RBISCO enzyme. It also increases the photosynthesis activity of the plant by increasing the activity of Rubisco (Delfine et al., 2005). Free sugar contents were significantly influenced by humic acid and ethanol and the highest free sugars were obtained from 800 mg/L humic acid application and 15% ethanol in foliar application. Foliar application of humic acid and ethanol increased proline content and accumulation of proline is a main factor that supports plants to sustain growth under stress and decreased osmotic potential, thereupon protecting cell turgor and water potentials for plant development (Hasegawa et al., 2000). Therefore, foliar spraying of humic acid and ethanol may decrease the effects of stress on plants.

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