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Original Article

Effects of Foliar Applications of Various Zinc to Improve Estragol (Methyl Chavicol), Biological Activities and Introducing New Monoterpene Oxygenated of Horehound (*Marrubium vulgare* L.)

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ABSTRACT

Marrubium vulgare (horehound) has been used to treat gastrointestinal disorders, inflammation. This plant has beneficial therapeutic properties and purifies the blood. In this research the changes in the composition of essential oils, aerial herb yield, and antioxidant activity of the aerial parts of *M. vulgare* were determined at different treatments of zinc. An experiment was performed using completely randomized design (CRD) with five different treatments (T1= Control, T2 = Zn sulfate, T3 = Nano Zn, T4 = EDTA- chelated Zn and T5 = Citrate chelated- Zn) in three replications in Eram Garden greenhouse, Shiraz, Iran. The essential oil of air-dried samples was obtained by hydro distillation and analysed by gas chromatography/mass spectrometry (GC/MS). The result showed a significant difference between all zinc treatments for Estragol (methyl chavicol), biological activities and introducing new monoterpene oxygenated. The highest value of methyl chavicol was observed in zinc citrate (68.2%), followed by EDTA chelated Zn (51.2%), Zn sulfate (40.6%) and Nano Zn (18.5%), respectively. The highest essential oil (EO) content (w/w%) was obtained in the plants treated with Nano Zn (0.25%). The high antioxidant activity (735.3 mg/L) was found when Nano-Zn was applied. Results revealed that application of Zn to horehound may be very useful for the production of active natural compounds such as methyl chavicol for drug industries and medical materials.

Abbreviations: DPPH: (2,2-diphenyl-1-picryl-hydrazyl-hydrate), EO: Essential oil, GC/MS: Gas chromatography/ mass spectrometry, HEO: Horehound essential oil.

INTRODUCTION

Horehound (*Marrubium vulgare* L.) is a flowering plant of the mint family, native to Europe, North Africa and Asia, and cultivated in various parts of the world [1]. Horehound is an important medicinal plant that is widely used in folk medicine and herbal medicine around the world [2]. Horehound Eos (HEOs) and their principal compounds are used because of their antimicrobial activity against a wide range of bacteria and fungi. In addition, drugs derived from horehound have been used to treat various disorders such as chronic cough, stomach, liver, heart, skin and immune system [3-5]. The compounds identified in this plant have been very diverse in the world and Iran. Germacrene D in southeastern Iran was the most important compound of essential oil, while β-caryophyllene was identified as the most important compound in central Iran, β-bisabolene in northern Iran [3-7]. Another natural compound were identified to be main compounds as δ-cadinene; isocaryophyllene; β-citronellol; (E)-β-farnesene; 1,8-cineol; geranyl formate ; α-pinene ; δ-eudesmol; γ-cadinene ; geranyl ; germacrene D-4-ol and citronellyl formate [5, 6].
Recently, researchers have shown that the diversity of medicinal compounds is attributed to genetic and external factors such as environmental conditions, agriculture and Fertilizer management (nutrient: N, Fe, Zn, …) [5,6]. The effect of micro on medicinal and aromatic plants is not well known [6]. Recently, trace elements have been proved to have a key role in forming active chemical compounds in medicinal

plants [7]. Zinc is an essential trace element that is also classified as heavy metal and has many structural and functional roles in plant metabolic processes, but both its deficiency and excess in the soil is considered as a limiting factor for plant growth [8]. On the other hand, foliar spraying is one of the most effective ways to provide the trace elements needed by plants [9].

Zinc acts as an activator and cofactor of some vital plant enzymes like anhydrase, carbonic dehydrogenase, alkaline phosphatase, and phospholipase, and RNA polymerase. This element also has a part in the construction of sugars, fats and proteins, plant photosynthesis as well as auxin biosynthesis as a growth-stimulating hormone [10, 11]. A large part of the earth in Iran are poor in micronutrients such as zinc due to being highly calcareous. Zinc deficiency is also widespread in soils with alkaline pH, which is specific to calcareous soils in Iran. The use of artificial chelates in plants is known as the most common method in agriculture, however, due to high costs and side effects and the environment, they cannot be used sustainably [12,13].

Nanomaterial is practically used in modern life in many fields, including agriculture and the environment. Today, researchers in the field of soil and plant nutrition have made great use of Nano-fertilizers to achieve sustainable development of agricultural products with minimal environmental degradation effects. [14, 15]. Nano fertilizers can compensate for plant nutrient deficiencies by increasing protein synthesis, carbohydrate and nitrogen metabolism [16, 15].

Foliar nutrition can reduce the stabilization of chemical fertilizers in the soil and thus reduce environmental dangers, including reducing soil and water pollution [10]. Leaf feeding is also important when there is an antagonistic phenomenon for the transfer of certain substances through the roots or the addition of substances to the soil that kill soil organisms [11]. With this optimal method, the elements can be provided to the plant in the fastest time, and also leaf nutrition can be effective in plant growth and increase their yield.

In practice, Iranian farmers in arid regions use chelates of trace elements in plant growth and development. The use of Nano-fertilizers in medicinal plants has been very low. To close the information gap, we investigated the effect of foliar application of zinc Nano-complex in comparison with zinc chelates and zinc citrate in some of the biological activities of essential oil (BAEO). This was an attempt to generate new information on the effectiveness of nano zinc fertilizers in the yield of medicinal plants and to develop a technical approach to the agricultural applications of nanomaterial. The present work aimed at evaluating the changes in antioxidant capacity and important chemical compounds of *M. vulgare* L. against the foliar application of different sources of zinc. The present work may be very useful for the production of active natural compounds such as methyl chavicol for drug industries and medical materials.

MATERIALS AND METHODS

Research Method

In order to study of the effect of different treatments of zinc on composition of essential oils and antioxidant activity of the aerial parts of *M. vulgare* a completely randomized design was conducted with three repetitions. Treatments consisted of Zn (T1 = Control, T2 = Zn sulfate, T3 = Nano Zn, T4 = EDTA- chelated Zn and T5 = Citrate chelated- Zn), spraying deionized water was performed in the control group.

Soil Analysis

The top soil utilized was loamy calcareous soil that dried at the air, homogenized, and sieved through a less than 2 mm sieve. Some physicochemical attributes of the soil were evaluated according to the following usual standard techniques: clay, sand, and silt fractions through hydrometer technique, saturated paste pH through glass electrode pH-meter; organic matter content (OM) through wet oxidation technique [12]; saturated extract electrical conductivity (EC) via EC-meter; plant-available manganese, total nitrogen (N) [13] (Table1).

Greenhouse Experiment

A greenhouse experiment with three replications was conducted in Eram Botanical Garden of Shiraz, Iran (36˚29′ N and 32˚52′ E, 1486 m above sea level). Two healthy seedlings of horehound were transferred to each pot. All pots included 2 kg of garden topsoil. For supplying essential nutrient elements at a sufficient level, all pots received 20 mg P/kg of soil as Ca $(H_2PO_4)_2.H_2O$, 10 and 5 mg Mn and Cu per kg soil, respectively, and 200 mg N/

kg soil as urea. The seedling pots irrigated every other day to near pot capacity for two periods of 30 and 50 days after transplanting. Zn was foliar used at the rate of 0.2% (w/v) from the mentioned sources of zinc. All zinc and nano-zinc complexes were visually similar and were fine white powders. The plants subjected to spraying with deionized water as control. Harvesting of plants was done 12 weeks after planting. Foliar application was done in two stages before the start of flowering with an interval of 7 to 10 days from each other. The herb materials were prepared for further laboratory analysis (Figs.1-3).

Table 1 Some physicochemical properties of soil in farm of Eram botanical garden.

pH	EC* (dS/m)	CEC (meq100/g)	CCE (mg kg)	OM (%)	T.N (%)	Soil texture	DTPA-extractable (mg/kg)			
							Zn	Fe	Cu	Mn
7.5	0.4	14	465	1.5	0.075	loam	0.85	2.34	1.12	4.4

*EC, electrical conductivity; CEC, cation exchange capacity; CCE, calcium carbonate equivalent; OM, organic matter; T.N, total nitrogen.

Fig. 1 Samples of *M. vulgare* L. in greenhouse of Eram Botanical Garden of Shiraz, Iran

Fig. 2 Samples of *M. vulgare* L. after foliar spray fertilizers

Fig. 3 Samples of *M. vulgare* L. at harvesting stage

Antioxidant Action

Antioxidant action of the essential oil were evaluated based on the radical removing impact of fixed DPPH free radical [17]. Twenty microliters of different concentrations of the essential oil samples in methanol (12.5 – 3200 µg/mL were mixed to 200 µL methanol solution of DPPH. The combination was permitted to stand at room temperature for 30 min in a dark place. The absorption of the samples was performed at a wavelength of 515 nm with ELx808 Microplate Spectrophotometer. The same amounts of DPPH and methanol were used as standard and blank, respectively. The scavenging activity was calculated using the following equation:

$$\text{Scavenging (\%)} = (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100$$

Where;

A_{sample} is the absorbance of the test sample and $A_{control}$ is the absorbance of the control.

The antioxidant activity of sample was formulated of IC50 (The half maximal inhibitory concentration). The graph was designed showing inhibition percentage vis-vis oil concentration to determine the IC50 value [17].

Essential oil (EO) preparation

The plant samples were shade dried at room temperature for 14 days (20-25°C). The hydro distillation was used to separate the essence of dried out samples for 3 h via the Clevenger-type [15].

Procedure of Oil Analysis

Analysis and identification of essential oils of horehound were performed via GC-MS. Analytical GC was used in gas chromatography with specifications [16].

Recognition of Essence Composites

The constituents of the horehound essence were recognized through calculating their RT (retention times) in specially planned temperature settings for n-alkanes: C8-C25, and the similar chromatographic settings were considered for horehound oil in an HP-5 column. Different composites were recognized accurately and their Figure ranges were compared by those from the interior position Figure range library or by valid composites or by those of described in the literature. The relative area percentage of flame ionization detector (FID) was used to quantify the essential oil compounds [16].

Statistical analysis

Data analysis (between zinc treatments and for antioxidant traits and important essential oil compounds) was conducted using ANOVA in SPSS software (v.25.0). Duncan's Multiple Range Test was used to compare the differences between groups ($p < 0.05$) between treatments.

RESULTS AND DISCUSSION

Antioxidant Activity

The mean values of IC50 (mg/L) DPPH assay in *M. vulgare* L. for each treatment are shown in Table 2. IC50 is a suitable measure of oxidation development in the oils. Thus, it was considered as a good index for the effective assessment of the antioxidant. To show antioxidant activity, Gallic acid was used as a standard. With stronger antioxidants, the IC50 was closer to the Gallic acid IC50. According to the results, Zn increased the amounts of inhibitory effects. So, the IC50 of extracts significantly decreased compared to the control group when using T3=Nano Zn, T4= EDTA- chelated Zn, respectively, to the horehound (Table 2). The results revealed that the antioxidant action of *M. vulgare* was improved after using Zn, especially Nano-Zn. The highest antioxidant capability was detected using Nano Zn (IC50: 735.3 mg/L), Because the closer IC50 is to IC50 gallic acid and the smaller its number, the stronger the antioxidant activity. According to our findings, other researchers have shown that Nano-zinc in peanuts, soybeans and anise increased antioxidant activity [18, 19, 20]. Marschner et al. proved that zinc acts as a co-factor and stimulator of antioxidant enzymes [21]. Antioxidant activity has also been attributed to various mechanisms, including binding of transition metal ion catalysts, prevention of continuous hydrogen accumulation, decomposition of peroxides, regenerative capacity, and radical inhibition [17].

On the other hand, the soils are globally poor in zinc (Zn), herbs cannot accumulate sufficient Zn in eatable parts that can meet the human nutrition need. The nano-Zn development can lead to new applications in soil science and plant biotechnology and the deficiency in plants can be eliminated by spraying this element.

Yields of Essential Oil and Natural Product Compound

The efficiency of essential oil (EO) was investigated under the influence of different sources of zinc and the results are shown in Table 2. Regardless of the type of zinc fertilizer, foliar application of zinc contributed to a significant increase in EO yield compared to the control (Table 2). EO yield values ranged from 0.20 ± 0.01% in control to 0.25 ± 0.01% in nano Zn, respectively. Moreover, differences between treatments Nano Zn (T3), EDTA chelated Zn (T4) and Zn sulfate (T2) were significant ($p < 0.05$). The maximum EO content was registered in T3 (0.25±0.01 %), followed by T4 and T5 (0.23 ± 0.07%); Treatments T1 and T2 had no significant differences.

We have found evidence of the importance of zinc nanoparticles on the performance of essential oils in *M. vulgare*. On the other hand, Nanoparticles showed higher reactivity due to the higher density of reactive areas, larger specific surface area, and increased reactivity of such regions on the particle’s surfaces. These properties simplify the absorption of fertilizers, which are created in Nano-scale [22].

The constituent of EOs was analyzed and concentrations of chemical compounds were detected (Table 3). The main constituents of essential oil (EO) varied at different concentrations between different treatments tested. Identified constituents amount to 96.2% and 99.8% of the total oil compound. Other researchers have reported that the number of major constituents of this plant is generally between 2 and 6, while others have shown other constituents at levels less than 0.05% [2]. Also identified constituents quantity to 57.50 -100% of the total oil compounds [23, 24, 256].

The main essence constituents of *M. vulgare* in control (T1) were recognized by a high content of geranial (41.6%) followed by neral (26.6%), methane thioamide (12.5%), methyl chavicol

(5.٤%), 2-aminopyrazine (1.5%) (Table 3). Application of T2 (Zn sulfate) improved these constituents to methyl chavicol (40.6%), 2-acetylpyridine thiosemicarbazone (31.9%) and new compound: 1,2,6-trimethyl-4(1H)-pyridinone (11.8%) and cholesta-7,9(11)-dien-3-ol, cholesta-7,9-dien-3-beta-ol (14.3%) significantly. Using Nano zinc (T3) treatment, the natural compound was geranial (24.7%), methyl chavicol (18.5%), neral (17.3%) and new compounds: benzyl alcohole (8.9%) and 1-deuterioformyl-2-methoxybenzene (7.0%). In treatments T4 (EDTA chelated Zn) and T5 (Citrate chelated Zn) of zinc application, 99.8% of the total compounds were identified, which included methyl chavicol and important new compounds. It was very interesting that, in EDTA zinc chelate treatment, methyl chavicol up to 9.5 times (from 5.4% in control to 51.2%) and in zinc citrate treatment (T5), this important drug combination showed up to 12.6 times a significant increase compared to the control (from 5.4% in control to 68.2%) (Fig. 4).

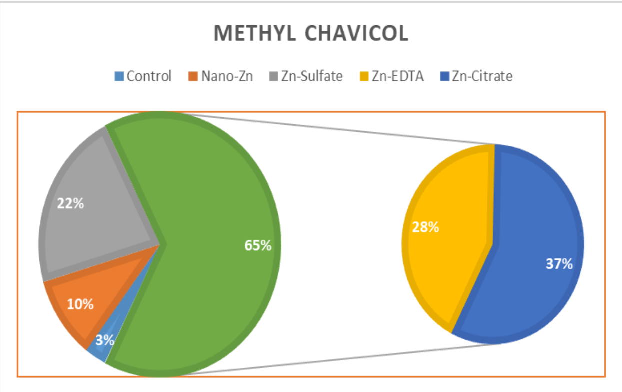

Fig. 4 The methyl chavicol content in the essential oil of *M. vulgare* in response to different sources of Zinc.

The special feature of *M. vulgare* (horehound) is that this plant has different essential oil compositions according to the climatic conditions of each region (The plant materials of this research were prepared from Eram Garden in Shiraz in Iran and the plants follow this law). For example, in the sample of horehound plants collected from the southeast of Iran "germacrene D "and for plants in the north and center of Iran "β-caryophyllene" the main composition of the essential oil has been identified [3, 26, 27, 24].

On the other hand, in different countries and regions of the world, the diversity of the main components of the essential oil of *M. vulgare* has been seen. In species grown in Egypt carvacrol and thymol were identified as important medicinal compounds [4, 28], in Slovakia, β-caryophyllene and germacrene D and in other countries E-methyl communate, 1,8-cineol, (E) -β-farnesene, carvacrol were identified as important medicinal compounds of this plant [2, 26].

According to research, the reason for this diversity of compounds is not yet clear. It may be related to the geographical or eco-physiological location of the area. However, the differences between the important pharmaceutical compounds of *M. vulgare* in different parts of the world have been proven [2] consistent with the results of other researchers, in this study, which was conducted in the southern region of Iran, seven new compounds of essential oil of *M. vulgare* was identified.

On the other hand, the researchers showed a relatedness between secondary metabolites and external factors, especially microelements, as well as the effect of the zinc on aroma, flavor and medicinal properties of plants [27, 28]. Zinc has a very beneficial effect on plant growth, photosynthesis, quantity and quality of essential oils, stimulating role of enzyme activity and biosynthesis of terpenes [29, 30, 31].

Although the impacts of foliar use of zinc in essential oil composition have not been studied in this plant, the following researches are available in the plants of the lamiaceae family (Mint): According to 24], the addition of Zn decreased eugenol in *Ocimum gratissimum* L. Also, a significant decrease in the percentage of α-Copan and germacrene D content was observed by adding zinc to the soil [20]. In this study, with foliar spraying, zinc treatments neral and geranial content were significantly reduced.

On the other hand, we observed a very interesting trend in the important drug combination of methyl chavicol, so that the amount of this compound showed a significant increase in several times in all zinc treatments. The highest increase in methyl chavicol was observed in zinc citrate treatment (68.2%) and followed by EDTA chelated Zn (51.2%), Zn sulfate (40.6%) and Nano Zn (18.5%) treatments showed a multiplication of this compound (Table 4).

Table 2 Mean value of IC50 (mg/L) DPPH assay and essential oil yield in *M. vulgare* L. in response to different sources of Zinc.

Zinc Treatments	IC50 (mg/L) Mean±SD	R^2	Essential oil yield %
control	776.2±4.16 b	0.99	0.20±0.01 c
Nano Zn	735.3±2.79 c	0.98	0.21±0.01 c
Zn sulfate	792.1±2.05 a	0.98	0.25±0.01 a
EDTA chelated Zn	739.4±2.20 c	0.97	0.23±0.01 b
Citrate chelated Zn	801.5±3.84 a	0.99	0.23±0.07 b

Different letters within the same row indicate mean values statistically different at $p < 0.05$ by Duncan test.

Table 3 Effect of different Zn fertilizer sources on concentrations of chemical compounds (%) identified in essential oil of *M. vulgare* L.

Compounds	RI[a]	Control Area,%	Zn sulfate Area,%	Nano Zn Area,%	EDTA chelated Zn Area,%	Citrate cheated Zn Area,%
Methyl chavicol	14.22	5.4±0.08667e *	40.6±0.3624 c	18.5±0.7081 d	51.2±0.2947b	68.2±0.4967 a
Unknown	15.61	7.1	-	-	-	-
Neral	16.71	26.6±0.57831 a	-	17.3±0.32590 b	-	-
Geranial	17.63	41.6±1.3707 a	-	24.7±0.5071 b	-	-
1,2,6-Trimethyl-4(1H)-pyridinone	30.48	-	11.8	-	-	-
1-Methoxy-1-propene	30.50	-	-	-	-	12.2
Unknown	30.55	-	-	-	-	19.4
Unknown	30.56	-	-	-	19.5	-
1-Deuterioformyl-2-methoxybenzene	30.57	-	-	7.0±1.3856 b	22.5±3.19764 a	-
Methane thioamide	30.59	12.5	-	-	-	-
Thiophene	30.59	-	-	-	6.6	-
Cholesta-7,9(11)-dien-3-ol, Cholesta-7,9-dien-3-beta-ol	30.60	-	14.3	-	-	-
Benzyl alcohol	30.62	-	-	8.9	-	-
Unknown	31.07	1.0±0.02309b	-	22.3±0.25482a	-	-
2-Aminopyrazine	32.71	1.5	-	-	-	-
2-Acetylpyridine thiosemicarbazone	32.78	0.5±0.00606b	31.9±0.06960a	-	-	-
Total Compounds %		96.2%	98.6%	98.7%	99.8%	99.8%

Data are mean ± standard deviation of three replications.

RT[*]: Retention time;

* Different letters within the same row indicate mean values statistically different at $p < 0.05$ as determined by Duncan test

Findings of this research confirm the results of Moghimipour et al [20]. They also showed an increase in germacrine D, (E) -karyofylene and the amount of volatile basil oils by zinc foliar application [21].

This also in agreement with previous findings that showed the levels of geraninal, eugenol, β-cubebene, (E)-caryophyllene in essence of basil were significantly increased by spraying of Nano complex and biochar [22].

Therefore, according to the results of this study, which showed a multiplication of methyl chavicol in all zinc treatments, the element zinc can be introduced to increase methyl chavicol in plants with this compound. To discover better effectiveness, additional research is needed on other plants.

CONCLUSION

For the first time, this study identified seven new drug combinations in *M. vulgare* L. from southern Iran, the most important of which were: geranial, neral, methane thioamide and methyl chavicol. The second part of our work showed the positive effect of zinc sources on horehound.

The foliar application of the zinc significantly enhanced some natural compounds contents, herbal yield and also the antioxidant activity of the horehound in all of the treatments, it is recommended to cultivate it in the agronomic regions with great Zn that unsuitable for producing most agricultural products. A significant increase in the important natural composition was observed with zinc treatment. The present work provides pharmacological and important implications because of the biological properties of these natural compounds and the industrial and medicinal benefits of these plants. Sometimes, Zn usage in helpful herbal for example horehound may be developed as an auspicious policy for producing more preferred pharmaceutically active compounds such as methyl chavicol for drug industries and medical materials.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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