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Effect of organic and inorganic zinc foliar application on the natural product composition and antioxidant activity of lemon balm (*Melissa officinalis*)

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ABSTRACT - Lemon balm (*Melissa officinalis* L.) is known as one of the most important hormonal plants (anti-thyroid), sedative, wound healing agents, and flavorings in the food industry. The present work aimed to determine the effect of different zinc (Zn) sources on the natural product composition, and antioxidant activity of lemon balm. The experiments were performed in a greenhouse in Eram Garden (Shiraz, Iran) with three replications and five different treatments including T₁: No zinc (control), T₂: EDTA-chelated Zn, T₃: nano Zn, T₄: Zn sulfate, and T₅: citrate-chelated Zn. The results showed that all Zn resources improved the chemical composition of the lemon balm essential oil (EO), such as geranial, caryophyllene oxide, and phthalic acid. The highest amount of geranial (40.8%) was achieved in the plants treated with citrate-chelated Zn. The highest total content of caryophyllene oxide (8.8%) was achieved when Zn sulfate was applied. The highest phthalic acid (2.24%) was obtained in the plants treated with Zn sulfate and nano Zn. The highest antioxidant activity (162.86 mg L⁻¹) was found when nano Zn was applied. The results revealed that the application of Zn to therapeutic plants like lemon balm could be very useful for the production of active natural compounds such as geranial, phthalic acid, and caryophyllene oxide for use in drug industries and medical materials.

INTRODUCTION

Melissa officinalis (lemon balm) is a plant belonging to the Lamiaceae family that has many uses as a flavoring in food and drink products and in traditional medicine. It is a fragrant (lemony) perennial plant with a height of about 1 m that grows in Western Asia, the Mediterranean, North Africa, and Southwestern Siberia. Lemon balm is a key plant among the native pharmaceutical plants of the genus *Melissa* (Saeb et al., 2011). *M. officinalis* has been utilized in traditional medicine, particularly in Iran, for treating certain health conditions such as headaches, nervousness, and gastrointestinal disorders (Saeb et al., 2011). Its leaves are believed to have carminative, antispasmodic, digestive, sedative, anesthetic, diuretic, and tonic properties. The extracts and essential oils of the plant contain antioxidant compounds (Erdemoglu et al., 2006), and they have the potential to be used as antimicrobial and antiviral agents (Saeb et al., 2011). It

has been reported that *M. officinalis* contains flavonoid and phenolic compounds, including rosmarinic acid (Herodež et al., 2003). Flavonoids have antioxidant properties that are attributed to their phenolic structure (Chang et al., 2002). The antioxidant properties of the compounds reported in *M. officinalis* are responsible for its therapeutic effects (Miraj et al., 2017).

Until a few years ago, the effects of microelements on medicinal and aromatic plants were not well known (Abugassa et al., 2008). Recently, trace elements have been shown to have a key role in forming active chemical compounds in medicinal plants (Murch et al., 2003). Zn is an essential trace element that is also classified as heavy metal and has many structural and functional roles in plants' metabolic processes, but its deficiency and excess in soil are considered as limiting factors for plant growth (Grejtovský et al., 2006).

Zn acts as an activator and cofactor of some vital plant enzymes like anhydrase, carbonic dehydrogenase, alkaline phosphatase, phospholipase, and RNA polymerase.



This element is also involved in the construction of sugars, fats, and proteins; plant photosynthesis; and auxin biosynthesis as a growth-stimulating hormone (Cakmak, 2008; Figueiredo et al., 2012).

A large part of the land in Iran is poor in micronutrients such as Zn due to its highly calcareous nature. Zn deficiency is also widespread in soils with an alkaline pH, such as the calcareous soils in Iran. It has been shown that foliar spraying is one of the most effective ways to provide the trace elements needed by plants (Baibordi and Malakouti, 2005).

Foliar nutrition can reduce the stabilization of chemical fertilizers in the soil and, thus, reduce environmental dangers including soil and water pollution (Malakuti and Tehrani, 1999). Leaf feeding is also important when an antagonistic phenomenon is involved in the transfer of certain substances through the roots to plants or when the substances, that kill soil organisms, are added to the soil (Kannan, 2010). With this feeding method, elements can be provided to the plant in the fastest time possible, and leaf nutrition can be effective in plant growth and increase yield. The present work aimed to evaluate the changes in antioxidant capacity and the important chemical compounds of *M. officinalis* L. against the foliar application of different sources of Zn. The present work could be very useful for the production of active natural compounds such as geranial, phthalic acid, and caryophyllene oxide for use in drug industries and medical materials.

MATERIALS AND METHODS

Soil characteristics analysis before planting

The utilized topsoil was loamy calcareous soil that was dried in the air, homogenized, and sieved through a less than 2-mm sieve. Some physicochemical attributes of the soil were evaluated according to the following standard techniques: clay, sand, and silt fractions through hydrometer technique; saturated paste pH through a glass electrode pH-meter; organic matter content (OM) through a wet oxidation technique (Nelson and Sommers, 1996); saturated extract electrical conductivity (EC) via an EC-meter; DTPA-extractable of Zn, iron (Fe), copper (Cu) and manganese (Mn); and total nitrogen (N) (Bremner, 1996) (Table 1).

Experimental design

A completely randomized design with three repetitions was utilized in this experiment. Treatments consisted of adding Zn at a rate of 0.2% (w/v) from different sources

of Zn (Fig. 1). Spraying of deionized water was performed in the control group.

Plant culture circumstances

A greenhouse test with three replications was conducted at the Eram Botanical Garden in Shiraz, Iran (39° 29' N and 33° 52' E, 1486 m above sea level). Each pot included 2 kg of the topsoil mentioned above. It was important that all pots were supplemented with sufficient levels of essential nutrient elements. Therefore, each pot received 20 mg P kg⁻¹ soil as Ca (H₂PO₄)₂H₂O, 10 and 5 mg Mn, and Cu kg⁻¹ soil, respectively, as their sulfates, and 200 mg N kg⁻¹ soil as urea. The prepared seedlings of *M. officinalis* L. were planted in the pots and irrigated with water every other day to near pot capacity. Zn was used as a foliar application twice (30 and 50 days after transplanting). each time at a rate of 0.2% (w/v) from the aforementioned sources of Zn (Fig. 1). The plants subjected to spraying with deionized water were considered as the control specimens. Plants were harvested 12 weeks after planting in the full flowering stage. The shoots of tested plants were prepared for further laboratory analysis.

Antioxidant action

The antioxidant action of the essential oil was evaluated based on the radical removing impact of fixed 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Twenty microliters of each different concentration of the essential oil samples in methanol (12.5 – 3200 µg mL⁻¹) were mixed with 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 samples was performed at a wavelength of 515 nm with an ELx808 Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The same amounts of DPPH and methanol without essential oil were used as standard and blank samples, respectively. The scavenging activity was calculated using the following equation (Gogoi et al., 2021):

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

where A_{sample} is the absorbance of the test sample and A_{control} is the absorbance of the control sample. The antioxidant activity of the sample was formulated of IC50 (concentration at which 50 % of DPPH radical formation gets inhibited). The graph was designed to show the inhibition percentage vis-vis oil concentration to determine the IC50 value as discussed by Najafian and Zahedifar (2015).

Table 1. Some physicochemical properties of soil in the farm of Eram botanical garden, Shiraz, used in this study

pH	EC* (dS m ⁻¹)	CEC (meq100g ⁻¹)	CCE (mg kg ⁻¹)	OM (%)	T.N (%)	Soil texture class	DTPA-extractable (mg kg ⁻¹)			
							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; Zn, zinc; Fe, iron; Cu, copper; Mn, manganese.

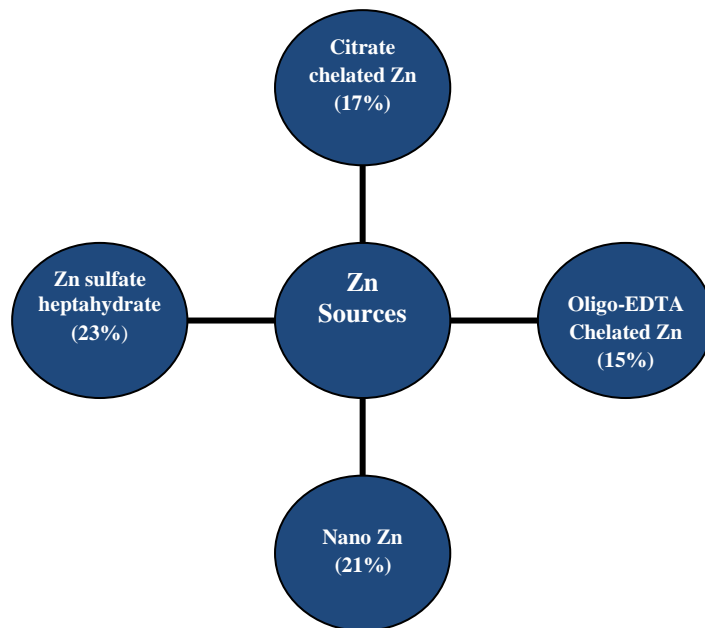


Fig. 1. Different sources of Zn that used in the present study. The percentage in parentheses of each Fig. partition indicates the amount of Zn in each corresponding compound.

Essential oil (EO) preparation

Shading of the vegetables was performed at ambient temperature (20-25 °C). The hydrodistillation was used to separate the essence of dried-out samples for 3 h via the Clevenger-type (Zahedifar and Najafian, 2015).

Essential oil analysis

The analysis and identification of EOs of lemon balm were performed via Gas chromatography-mass spectrometry (GC-MS). Analytical GC was used in gas chromatography with the “Agilent with typical 7890 A and equipped with a flame ionization detector (FID” specifications. A molten silica column with the following specifications was also employed. Agilent HP-5 with fused silica column; 5% phenyl methyl polysiloxane (30 meters × 0.32 millimeters), with a film thickness of 0.25 micrometers; it also had an Agilent chemo station software structure. The size of the sample inserted into the GC was 0.2 microliters of clean essential oil. The injector temperature was kept at 250 °C, and the detector temperature was maintained at 280 °C. A flow rate of 1 milliliter per minute was also used for nitrogen gas. The oven heat program ranged from 60 °C to 210 °C at a speed of 4 °C per minute and was then programmed with a temperature of 20 °C per minute to 240 °C before finally being kept at the same temperature for 8.5 minutes. The split ratio was 1/50.

Recognition of essential oil composites

The constituents of the lemon balm essence were recognized by calculating their retention indices (RIs) in specially planned temperature settings for n-alkanes: C8-C25. Similar chromatographic settings were

considered for lemon balm oil in an HP-5 column. Different composites were recognized accurately, and their figure ranges were compared with those from the interior position figure range library, valid composites, or the literature (Adams, 2007; Afshar et al., 2021; Farahbakhsh et al., 2020; Najafian and Zahedifar, 2018). The relative area percentage of FID was used to quantify the essential oil compounds.

Statistical analysis

Data analysis was conducted using ANOVA in SPSS software (v. 25.0). Duncan’s Multiple Range Test (DMRT) was used to measure the significant differences ($P < 0.05$) between treatments.

RESULTS AND DISCUSSION

EO compound

The lemon balm’s volatile oil contained 37 composites (Table 2). Total compounds were recognized in the following treatments: T₁ (98.87%), T₂ (99.39%), T₃ (100%), T₄ (99.77%), and T₅ (99.97%) (T₁: Control, T₂: Zn sulfate, T₃: nano Zn, T₄: EDTA-chelated Zn, T₅: Citrate-chelated Zn). The major volatile oil constituents of *M. officinalis* were determined with a higher amount of Neral (24.52%) followed by *trans*-citril (24.03%), *trans*-caryophyllene (17.46%), geranial (10.78%), 1-naphthalenol (3.15%), and caryophyllene oxide (2.37%) (T₁). The use of Zn fertilizers led to considerable variations in some components of volatile oil. The most reductions in the proportions of *trans*-caryophyllene in T₂ (7.13%), T₃ (7.12%), and T₅ (16.73%) treatments were noticed when compared to the control specimens (17.46%).

Table 2. Effect of different Zn fertilizer sources on concentrations of natural compounds (%) identified in essential oil of *Melissa officinalis*.

Compounds	RT*	T ₁ **	T ₂	T ₃	T ₄	T ₅
β-Pinene	4.99	0.11	-	-	-	-
1,5,8-p-menthatriene	5.71	-	1.52	1.52	-	-
(2-Methylprop-1-enyl)- cyclohexa-1,5-diene	6.44	-	1.32	1.32	-	-
Benzene, 1-methyl-2-(1- methylethyl)-	6.95	-	4.64	4.64	-	-
3-Octanone	7.72	0.06	-	-	-	-
2,3- Dihydroxypropionaldehyde	7.80	-	2.98	2.8	-	-
Propanal	7.86	-	2.0	-	-	-
Benzene, 2-ethenyl-1,4-dimethyl-	-	-	1.4	-	-	-
1-Octen- 3-ol	8.43	0.29	-	-	-	0.25
6-Methyl-5-hepten-2-one	8.64	0.45	-	-	-	0.46
Rosefuran	9.051	0.21	-	-	-	-
Perillen	9.281	0.07	-	-	-	-
Linalool	11.13	1.10	-	-	-	0.57
Benzenemethanol, 4-amino	11.60	0.17	-	-	-	-
Citronellal	12.29	0.12	-	-	-	0.96
Trifluoroacetyl-lavandulol	12.43	0.30	-	-	-	-
1,5-Heptadiene	12.74	0.35	-	-	-	0.19
Cyclohexane	13.01	1.02±0.01b	-	1.65±0.03a	-	0.71±0.06c
trans-chrysanthemal	13.21	0.86±0.03a	-	-	-	0.28±0.01b
α-Thujone	13.45	-	0.65	-	-	-
Cyclohexane, ethenyl	13.52	1.98	-	-	-	1.69
E,E-α-Farnesene	14.07	0.29	-	-	-	-
Methyl chavicol	14.20	-	-	-	6.08	-
Benzene, 1-methoxy-4-(2- propenyl)	14.21	-	1.2	1.19	-	-
β-elemene	14.95	0.36	-	-	-	-
Cyclopentane	15.59	-	-	-	10.74	-
trans-Caryophyllene	16.18	17.46±0.25a	7.13±0.18c	7.12±0.19c	-	16.73±0.15b
3,7-dimethyl-, methyl ester	16.34	0.58	-	-	-	0.54
Neral	17.12	24.52±0.28b	19.91±0.06c	19.91±0.06c	29.60±0.30a	29.92±0.23a
Nerol	17.28	-	3.14	3.14	-	-
2-Norpinene	17.45	0.40	-	-	-	-
cis-Geraniol	-	-	-	-	-	3.03
Geranial	17.87	10.78±0.12c	29.65±0.18b	29.65±0.18b	40.34±0.08a	40.78±0.13a
trans-Citral	18.18	24.03	-	-	-	-
α -Methylhexanonisoxim	20.46	-	0.80	-	-	-
Neric acid	22.34	0.22	-	-	-	-
Cyclohexane	22.62	0.12±0.003b	-	-	-	2.39±0.31a
Verbenone	23.28	-	0.63	0.63	-	-
Chrysanthenone	23.31	0.40	-	-	-	-
Caryophyllene oxide	23.94	2.37±0.19c	8.8±0.15a	8.0±0.037b	-	-
Caryophylla-4(12)	24.16	2.32±0.16a	-	0.42±0.01b	-	0.37±0.012b
δ-Selinene	24.41	-	2.82	2.82	-	-
γ -Cadinene	24.44	1.27	-	-	-	-
1-Naphthalenol	24.64	3.15±0.19b	-	-	5.31±0.16a	-
t-Muurolol	25.30	-	3.50	3.50	-	-
α.-Cadinol	25.35	1.34±0.20b	-	3.66±0.17a	-	0.67±0.30c
3-Methylene-bicyclo	25.99	-	1.40	1.40	-	-
undecan-5.β.-ol	26.03	0.66	-	-	-	-
Methyl 2-hydroxy-6-heptadec-8Z caryophylla-3,8(13)-dien-5.beta.- ol	26.43	-	0.70	0.70	-	-
26.46	0.25	-	-	0.46	-	-
Cyclohexane	26.89	-	0.40	0.40	-	-
Isoaromadendrene epoxide	26.92	0.50	-	-	-	-
Farnesol isomer A	27.82	0.18	-	1.04	-	-
5α-Ergost-8(14)-ene	30.07	-	1.64	-	-	-
(-)-Caryophyllene oxide	30.09	0.08	-	-	-	-
Tricosanoic acid	31.03	-	-	0.43	-	-
Benzo[1,2-a:4,3-a']diphenazine, 16,17-dihydro	31.06	-	-	0.30	-	-

Table 2. Continued

Compounds	RT*	T ₁ **	T ₂	T ₃	T ₄	T ₅
4H-1-Benzopyran-4-one	31.5	-	-	-	7.7	-
1,2-enenedicarboxylic acid	31.71	0.31	-	-	-	-
Bianthrone	33.08	-	0.94	0.94	-	-
Phthalic acid	33.87	0.18±0.01b	2.24±0.14a	2.24±0.14a	-	0.41±0.02b
Total Compounds %		98.87%	99.39%	100%	99.77%	99.97%

Data are mean ± standard deviation of three replications. RT*: Retention time; Zn (Zn) treatments: T₁** : Control, T₂: Zn sulfate and T₃: nano Zn, T₄: EDTA- chelated Zn, T₅: Citrate chelated Zn. Means followed by the same letter are not significantly different by DMRT ($P \leq 0.05$). - Not detected.

Neral was the second most abundant component, indicating the same trend. This component reached 24.52% in the control condition and 19.91% in the nano Zn and Zn sulfate conditions; meanwhile, it increased significantly in the EDTA-chelated Zn and citrate-chelated Zn conditions. Although the impacts of the foliar use of Zn in essential oil composition have not been studied in this plant, the following studies are available regarding the plants of the Lamiaceae family (mint). According to Bisht et al. (2019), the addition of Zn decreased eugenol in *Ocimum gratissimum* L. Also, significant decreases in the percentages of α -Copa and germacrene D content were observed after adding Zn to the soil (Bisht, et al., 2019). Geranial is another important component that has exhibited an interesting change. Compared to the control (10.78%), a significant increase was found in the geranial percentage when using Zn (Table 2). The proportion of geranial increased to 29.65, 29.6, 40.34, and 40.78% for T₂, T₃, T₄, and T₅, respectively.

Caryophyllene oxide also increased when using Zn. The amount of this constituent was 2.37% in the control condition and 8.0% and 8.8% after applying Zn to the T₃ and T₂ specimens, respectively. In line with the results of the current study, the addition of Zn led to an increase in the content of a very important compound (“E- Caryophyllene”) in the plants of the mint family (Bisht et al., 2019).

Phthalic acid was another significant natural component indicating an interesting change. According to Table 2, the quantity of phthalic acid substantially increased at the end of the test. The amount of this constituent at the control was 0.18%, and its proportions were 0.41, 2.24,

and 2.24% after the foliar application of T₅, T₃, and T₂, respectively.

It has been found that the accumulation of natural products in foliage depends on the relationships among several internal and external factors, especially the optimal level of micronutrients, particularly Zn (Srivastava et al., 2006). Researchers have shown that the use of Zn in plants causes the activity of glucosinolates, which may also affect the flavor and medicinal properties of plants (Coolong et al., 2004). The findings of the present research corroborate the results of Moghimipour et al. (2017). They also showed an increase in Germacrene D, E -Caryophyllene, and volatile basil oils after foliar Zn application (Moghimipour et al., 2017). These results are also in agreement with previous findings that showed the levels of geranial, eugenol, β -cubebene, and E-caryophyllene in essential of basil were significantly increased by spraying a nano complex and biochar (Najafian and Zahedifar, 2018).

Antioxidant activity

The mean values of IC₅₀ (mg L⁻¹) DPPH assay in *M. officinalis* for each treatment are shown in Fig. 2. IC₅₀ is a suitable measure of oxidation development in oils. Thus, it was considered as a good index for the effective assessment of the antioxidant. Gallic acid is used as a standard indicator of antioxidant activity. With stronger antioxidants, the IC₅₀ is closer to that of gallic acid. According to the results, Zn increased the inhibitory effects. Thus, the IC₅₀ of lemon balm extracts significantly decreased compared to the control group when using T₃, T₂, and T₄ (Fig. 2).

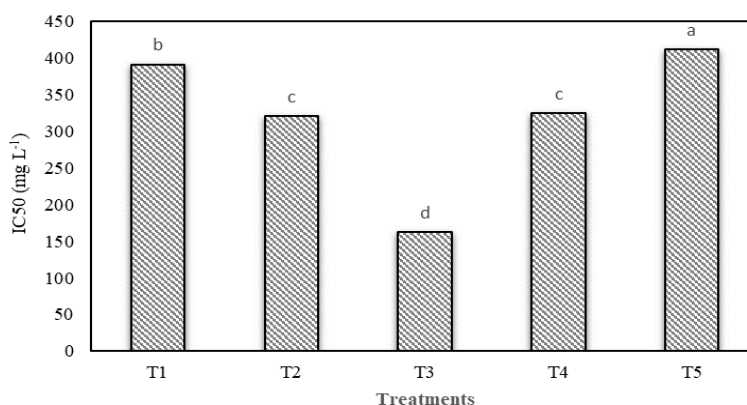


Fig. 2. Effect of different sources of Zn on the IC₅₀ of *Melissa officinalis* L. Means followed by the same letter are not significantly different by DMRT ($P \leq 0.05$). Data are mean ± standard deviation of three replications T₁: Control, T₂: Zn sulfate and T₃: nano Zn, T₄: EDTA- chelated Zn, T₅: Citrate chelated Zn.

The results revealed that the antioxidant action of *M. officinalis* L. improved after using Zn, especially nano Zn with IC50 of 162.86 mg L⁻¹ (Fig. 2). Since soils are globally poor in Zn, herbs cannot accumulate sufficient Zn in the eatable parts to meet human nutritional needs. However, nano Zn development can lead to new applications in soil science and plant biotechnology, and the abovementioned deficiency in plants can be eliminated by spraying this element as nano-particles.

CONCLUSIONS

The results of this study indicated that the foliar application of Zn significantly enhanced the levels of some natural compounds and the antioxidant activity of lemon balm in all of the treatments. Thus, it is recommended to cultivate lemon balm with Zn in agronomic regions which are unsuitable for producing most agricultural products. A significant increase (10.80 to 40.80%) in an important natural composition (geranial) was observed after Zn treatment was applied. The present work provided important pharmacological implications owing to the biological properties of these natural compounds and the industrial and medicinal benefits of these plants. According to the findings of this study, Zn is helpful for growing herbs such as lemon balm, which could be developed as an auspicious policy for producing more preferred pharmaceutically active compounds, such as geranial, for drug industries and medical materials.

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CONFLICT OF INTEREST

The authors declare no competing financial interest of this research.

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اثر محلول پاشی روی آلی و معدنی بر ترکیب فرآورده‌های طبیعی و فعالیت آنتی‌اکسیدانی بادرنجبویه (*Melissa officinalis*)

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ترکیبات تولیدی طبیعی

چکیده - بادرنجبویه (*Melissa officinalis* L.) به عنوان یکی از مهم ترین گیاهان هورمونی (ضد تیروئید)، آرام بخش، ترمیم کننده زخم و طعم دهنده در صنایع غذایی شناخته می شود. کار حاضر با هدف تعیین تأثیر منابع مختلف روی بر ترکیب فرآورده‌های طبیعی، عملکرد گیاه و فعالیت آنتی‌اکسیدانی بادرنجبویه انجام شد. آزمایش‌ها در گلخانه باغ ارم شیراز با ۳ تکرار و ۵ تیمار مختلف شامل T₁ بدون روی (شاهد)، T₂ روی کلاته با EDTA، T₃ نانو روی، T₄ سولفات روی و T₅ کلات سیترات روی انجام شد. نتایج نشان داد که تمام منابع روی باعث بهبود ترکیب شیمیایی اسانس بادرنجبویه مانند گرانپول، کاریوفیلین اکساید و فتالیک اسید شدند. محتویات اسانس (درصد وزنی) از ۰/۲۸٪ تا ۰/۴۴٪ متغیر بود. بیشترین مقدار گرانپول (۴۰/۸ درصد) در گیاهان تیمار شده با کلات سیترات روی به دست آمد. بیشترین مقدار کل کاریوفیلین اکساید (۸/۸ درصد) با استفاده از سولفات روی به دست آمد. بیشترین فتالیک اسید (۲/۲۴ درصد) در گیاهان تیمار شده با سولفات روی و نانو روی به دست آمد. بیشترین فعالیت آنتی‌اکسیدانی (۱۶۲/۸۶ میلی گرم در لیتر) در استفاده از نانو روی یافت شد. نتایج نشان داد که کاربرد روی در گیاهان درمانی مانند بادرنجبویه می تواند برای تولید ترکیبات طبیعی فعال مانند گرانپول، فتالیک اسید و کاریوفیلین اکساید برای استفاده در صنایع دارویی و مواد پزشکی بسیار مفید باشد.