ÇOMÜ Zir. Fak. Derg. (COMU J. Agric. Fac.)
2016: 4 (2): 75–85

Effects of Some Micronutrients on Antioxidant Activity of Thyme (*Thymus vulgaris* L.)

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Recieved: 03.01.2017 Accepted: 02.02.2017

Abstract

The species of the Lamiaceae family such as thyme possess appreciable antioxidant activity. The aim of trial was to study the effects of some micronutrients on antioxidant activity of thyme. The study was conducted at the Experimental Fields of Agronomy Department, Faculty of Agriculture and the Lab of Biology Department, Urmia University, West Azerbaijan, Iran, during 2015–2016. In the trial used randomized complete block design in three replications. Control, Fe, Zn, B and Mn were used as fertilizer treatments. Total phenolic content, total flavonoid content, DPPH radical scavenging activity, nitric oxide radical scavenging activity and Chain–breaking activity were determined. According to the results the effect of micronutrients in the first harvest on total phenols content, total flavonoids content, nitric oxide radical scavenging, DPPH radical scavenging activity and Chain–breaking activity was significant whereas in second harvest only nitric oxide radical scavenging activity was significant. In terms of total phenolic content, total flavonoid content, nitric oxide radical scavenging activity, DPPH radical scavenging activity, and Chain–breaking activity the best treatment was Mn, B, control and Mn respectively.
Keywords: Foliar Application, Micronutrients, Antioxidant Activity, Thyme.

Öz

Bazı Mikro Elementlerin Adi Kekiğin (*Thymus vulgaris* L.) Antioksidan Aktivitesi Üzerine Etkileri

Lamiaceae familyasının diğer türlerinde olduğu gibi adi kekik'te de antioksidan aktivite bulunmaktadır. Bu araştırmada mikro elementlerin adi kekiğin antioksidan aktivitesi üzerine olan etkisinin belirlenmesi amaçlanmıştır. Araştırma Iran'ın Batı Azerbaycan Eyaleti, Urumiye, Urumiye Üniversitesi, Ziraat Fakültesi Tarla Bitkileri Bölümü Deneme Tarlasında ve Biyoloji Bölümü Laboratuvarında 2015–2016 yıllarında yürütülmüştür. Deneme tesadüf blokları deneme desenine göre üç tekrarlamalı olarak kurulmuştur. Denemede Fe, Zn, B ve Mn gübre olarak kullanılmıştır. Toplam fenolik madde içeriği, toplam flavonoid içeriği, DPPH serbest radikalı giderme aktivitesi, nitrik oksit serbest radikalı giderme aktivitesi ve Zincir kırıcı aktivitesi gibi karakterler incelenmiştir. Verilerin varyans analizine göre ilk hasatta gübre uygulamaları arasında toplam fenolik madde içeriği, toplam flavonoid içeriği, DPPH serbest radikalı giderme aktivitesi, nitrik oksit serbest radikalı giderme aktivitesi ve Zincir kırıcı aktivitesi karakterleri bakımından farklılıklar önemli bulunmuştur. İlk hasada rağmen ikinci hasatta sadece nitrik oksit serbest radikalı giderme aktivitesi bakımından gübre uygulamaları arasında farklılıklar önemli görünmüştür. Toplam fenolik madde içeriği, toplam flavonoid içeriği, nitrik oksit serbest radikalı giderme aktivitesi, DPPH serbest radikalı giderme aktivitesi ve Zincir kırıcı aktivitesi bakımından sırasıyla Mn, B, kontrol ve Mn uygulamaları en iyi sonuçları ortaya koymuştur.
Anahtar Kelimeler: Adi Kekik, Antioksidan Aktivite, Mikro Elementler, Yaprak Uygulaması.

Introduction

Different free radical productions occur continuously in all plant and animal cells as part of usual cellular function. Excess different free radical productions originating from endogenous or exogenous causes might play a role in many diseases (Young and Woodside, 2001). Dissimilar antioxidant compounds play important roles in preventing formation of Reactive Oxygen Species in organisms. Produced synthetic antioxidants in recent years are risky and unsafe; because of their toxicity and carcinogenicity, considerable attention has been directed towards the identification of natural and safe antioxidants from plants such as some medicinal and aromatic plants (Gordon, 1996; Caia et al., 2004). Phenolic compounds which widely distributed in plants are secondary metabolites. These compounds are important components of many fruits and vegetables not only for their major influence on sensory qualities of the fruit, but also for their antioxidant, anti–carcinogenic, antimicrobial, and other medical properties (Alesiani et al., 2010). So, the role of fruits and vegetables

in prevention of some disease is partly related with the antioxidant properties of their constituent phenolic compounds (Scalbert and Williamson, 2000).

The genus *Thymus* which belongs to Lamiaceae family includes many species. The species *Thymus vulgaris* L. is a small woody shrub (10–30 cm) and native to the Mediterranean region. The plant is a medicinal and aromatic plant characterized by a broad chemical intraspecific variability (Bozin et al., 2006; Figueiredo et al., 2008). The leaves of the plant used fresh or dried as a spice to add a distinctive aroma and flavor to food (Lee et al., 2004). Nowadays different species of this genus is cultivated in large scale in Iran. Evidently, thyme continues to command an important place in expanding world market (Carlen et al., 2010). Widespread range of biological and pharmacological properties such as antiseptic, carminative, antimicrobial, and antioxidative there are in natural essential oils due to phenolic compounds (Bozin *et al.*, 2006; Nejad et al., 2008). Thymol and carvacrol as essential oil components reported to act as antioxidant activity (Jukic, and Milos, 2005). The compositions of the natural essential oils which extracted from aromatic plants are very much influenced by ecological factors, such as origin, climatic conditions, soil, and biotic intrinsic factors, such as species, cultivar, clone and ecotype, and technological factors, cultivation techniques, types of collection processes, storage conditions of raw materials and processing technologies (Russo et al., 2013). Some documents are dedicated to the antimicrobial activity of the essential oil of thyme and of its single constituents. Moreover, the antioxidant property of the plant makes its helpful for food safety (Amorati et al., 2013). Essential oil content of thyme has been reported from 0.32% to 4.9% (Carlen et al., 2010).

Micronutrients possess significant effectiveness on vegetative and generative phases of plants such as medicinal and aromatic plants (Heidari et al., 2008). Iron (Fe) is one of the three micro essential nutrient elements required by plants for example the ion is important in cytochrome structure. (Schonherr et al., 2005). Zinc (Zn) is an important micro element related with several enzymatic activities in all photosynthetic plants. The ion is essential in different vital enzymes and growth regulators (Babaeian et al., 2012; Samia and Mohmoud, 2009). Manganese (Mn) as an important micro element is involved in numerous biochemical functions, primarily acting as an activator of some enzymes such as dehydrogenases and decarboxylases involved in respiration, amino acids and lignin synthesis, and hormone concentrations (Younis et al., 2013). Boron (B) is one of the micronutrients which made resistance of plasmalemma in cells. The ion increase resistance combination by other minerals and necessary for plants (Widom and Mihalkovic, 2008). Some researcher indicated that there are relationship among micronutrients and essential oil contents and components. Misra et al., (2006) showed that essential oil biosynthesis and content in basil (*Ocimum sanctum* L.) as aromatic plant is strongly influenced by iron (Fe) and zinc (Zn). Akhtar et al., (2009) reported that Essential oil content of paper mint (*Mentha piperita*) improved by 28.2% by zinc (Zn) chloride foliar application compared with the control. Misra and Sharma (1991) indicated that zinc (Zn) application stimulated the fresh and dry matter production, essential oil content and menthol concentration of Japanese mint. Nassiri et al. (2010) showed that foliar application of iron (Fe) and zinc (Zn), increased flower yield, percentage and essential oil yield in chamomile. There are no papers have been written about the effect of micronutrients on antioxidant activity of thyme. It is believed that this study will be a good source for future. The main objective of the submitted work was to evaluate the effect of some micronutrients (Fe, Zn, B and Mn) on antioxidant activity of thyme under Urmia condition, West Azerbaijan, Iran.

The extracts of thyme were evaluated to determine the total amount of phenol, flavonoid, Chain–breaking activity (CBA), radical scavenging activity (DPPH) and Nitric oxide radical inhibition assay (NO°).

Materials and Methods

This study was conducted at the experimental fields of the Department of Agronomy, Faculty of Agriculture (latitude 37.53° N, 45.08° E, and 1320 meter above sea level) and the Lab of Biology Department, Urmia University, Urmia, Iran, during 2015–2016. The trial arranged in a randomized complete block design and three replications in plots of an area of 6 m^2. West Azerbaijan Province is located in the utmost end of Iran's northwest, between 35 degrees 58 minutes and 46 degrees northern Latitude, and also between 44 degrees 3 minutes and 47 degrees 23 minutes longitude. This province covers an area of 37614 km^2 which includes the 23 percent of the whole country's area (Najafi and Darvishzadeh Sherafatmand, 2013). The long term outdoors climatic data of the experimental city

(Table 1.) are showen. The land was plowed at the optimum moisture level (field capacity) and leveled. Phosphorus and Potassium fertilizers were used at pre–sowing in autumn, according to soil analysis and farrowed in 50 cm. The seeds for sowing were obtained from Turkey (population of Deutsche Welle, Germany). Sowing was carried out in green house at the Department of Horticulture, Faculty of Agriculture, Urmia University, during the period from 21. 03. 2015 till 06.05.2015. The seeds were sowed in plastic pots filled with soil, sand, and peat moss substrate as a material to germination. After sowing was irrigated regularly depending on weather conditions and development stage of plants. Seedlings were harvested and planted in the experimental field. Nitrogen fertilizer was used in planting time, and vegetative phase according to soil analysis. Irrigation was conducted depending on plants need. Foliar application of micronutrients included: control, Fe, Zn, B and Mn. Foliar application of micronutrients was done at three times: 1) at autumn in first year, 2) early of vegetative phase in second year, and 3) early of generative phase or flowering stage in second year. Harvestings were done in 50% flowering in the second year for two times.

Table 1. The long term outdoors climatic data of the experimental city*.

Months	Rainfall (mm)	Temperature (C°) (Average)	Temperature (C°) (Lowest)	Temperature (C°) (Highest)	Wind speed (Knots)
January	29.3	19.3	-22.8	16.4	2.0
February	33.2	13.4	-22.0	21.0	2.5
March	51.5	6.8	-19.0	26.0	3.3
April	61.3	1.3	-12.0	30.8	4.0
May	44.3	-1.8	-1.6	31.8	3.5
June	14.2	0.1	4.0	36.2	3.4
July	5.5	5.3	9.8	38.0	3.1
August	2.4	11.0	8.0	39.2	3.0
September	4.7	15.7	2.2	36.0	3.0
October	24.3	20.3	-5.0	30.0	2.6
November	39.6	23.9	-13.4	23.0	2.2
December	28.6	23.5	-20.0	21.4	2.0

Soil samples (0–30 cm) were taken in autumn before application of fertilizers. Soil analysis results of the experimental soil samples in the field (Table 2) are shown.

Table 2. Soil analyses results of the experimental soil samples in the field before corm sowing.

EC	1.32 dSm^{-1}	**O.C**	1.28%
$CaCO_3$	16.3%	**pH**	7.7
B.S	49%	**K**	320 mg kg-1
F.C	27%	**P**	9.54 mg kg-1
Clay	44%	**Fe**	17 mg kg-1
Loam	34%	**Zn**	1.6 mg kg-1
Sand	22%	**B**	0.4 mg kg-1
Texture	Clay-Loam	**Mn**	15 mg kg-1

Preparation of extracts

Fresh leaves of thyme were cut into small pieces and dried at room temperature in shadow. The dried plant materials were powdered with a grinder. Methanol used as extraction solvent. Extraction procedure involved the addition of 25 mL solvent to 2 g sample and shaking the samples for 60 min at low speed and then the extract was passed through Whatman filter paper No.1 (Whatman Ltd., England). Extraction was performed twice more with magnetic stirring for 60 min. The solutions were sealed andstored at 4 °C until experiments in the dark. Light exposure was avoided during the extraction process (Farnad et al., 2014).

Total phenolic content determination

Total phenolic contents (TPC) of extracts were estimated with the Folin–Ciocalteu colorimetric method described previously (Kahkonen et al., 1999) with a little modification. Folin Ciocalteu's phenol reagent (1 mL) and 10 % w/v Na2CO3 (1 mL) were added to sample extract (10 µl) and the mixture reaction was incubated in the dark for 60 min. The absorbance of the reaction

mixture was then measured at 750 nm. TPC were expressed in terms of g Gallic acid equivalents/ 100 g thymus vulgaris powder **(**The calibration equation for Gallic acid: y= 0.0415x- 0.0163).

Determination of total flavonoid content (TFC)

Total flavonoid contents (TFC) of extracts were estimated with aluminum chloride colorimetric method described previously (Youngjae *et al*., 2007) with a little modification. 10 µl of extract was diluted with 1 mL of deionized water. Then 0.075 mL of 5 % NaNO2 was added to this mixture, which was allowed to stand for 5 min at room temperature, and 0.15 mL of 10 % AlCl36H2O was added. The mixture was allowed to stand for 6 min at room temperature, and 0.5 mL of 1 mol/L NaOH was added, and the total volume was made up to 3 mL with deionized water. The absorbance of the solution was measured immediately at 510 nm. TFC were expressed in terms of g quercetin equivalents/100 g Peppermint powder **(**The calibration equation for Gallic acid: y= 0.0772x- 0.0084).

Determination of DPPH radical scavenging activity

The free radical scavenging activity of plant extracts were determined by slight medications of the method described previously (Hatano et al., 1988). 10 µl of the extract was added to a 2 mL of DPPH (1,1–diphenyl 2–picryl hydrazyl). The solution was incubated for 30 min in the dark at room temperature. After the incubation, the mixture absorbance was measured at 517 nm. The DPPH radical scavenging activity was calculated according to the following formula:

Percentage inhibition: [(A blank – A sample) / A blank] × 100

Determination of nitric oxide radical scavenging activity

Nitric oxide radical inhibition can be estimated by the use of Griess Ilosvay reaction (Garrat, 1964).In this investigation, Griess Ilosvay reagent is modified by using naphthyl ethylene diamine dihydrochloride (0.1 % w/v) instead of 1–naphthylamine (5 %). The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (0.5 mL) and thymus vulgaris leaves extracts (10 µl) was incubated at 25 C for 150 min. After incubation, 0.5 mL of the reaction mixture was mixed with 1 mL of sulfanilic acid reagent (0.33 % in 20 % glacial acetic acid) and allowed to stand for 5 min to complete diazotization. Then, 1 mL of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25 C. A pink colored chromophore was formed in diffused light. Gallic acid and ascorbic acid were used as positive controls. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. The nitric oxide radical scavenging activity was calculated according to the following formula:

%Nitric oxide scavenging activity = (A blank – A sample)* 100/ A sample

Chain–breaking activity (CBA)

The Chain–breaking activity was based on the method of Brand–Williams et al., (1995) with slight modification. The Chain–breaking activity was expressed by the reaction rate k and calculated by the following equation: Abs^{-3} - $Abs0^{-3}$ = -3kt

Where Abs0 is initial absorbance, Abs is absorbance at increasing time, (t), and the reaction rate was expressed as k. Antioxidant activity was reported as ($-Abs^{-3}$ /min/mg extract).

Statistical analysis

Experimental design was a randomized complete block design with three replications and five micronutrient treatments per each block. The analysis of variance (ANOVA, one–way analysis) was performed using SAS 9.1 (SAS Institute, Cary, North Carolina, USA) to detect the significance of differences among the treatment means. Mean comparison of traits was performed using Duncan (Saadat et al., 2014).

Results and Discussion

The results showed that the effect of micronutrients in the first harvest on total phenols content, total flavonoids content, nitric oxide radical scavenging, DPPH radical scavenging activity and Chain–breaking activity was significant ($P \leq 0.01$). And in second harvest, the effect of micronutrients on Nitric oxide radical scavenging activity was significant ($P \leq 0.01$).

Table 2. Effect of micronutrients and on antioxidant properties of thyme in first harvest.

		Mean Square				
Sov	**df**	**Total phenols content (mg gallic acid/g DW)**	**Total flavonoids content (mg quercetin/g DW)**	**Nitric oxide radical scavenging activity (%)**	**DPPH radical scavenging activity (%)**	**Chain–breaking activity**
Block	2	7.94*	0.003	77.46	89.13	143.33*
Micronutrients	4	44.17**	0.094**	618.57 **	284.74**	676.41**
Error	8	1.04	0.004	59.83	25.61	28.55
CV (%)	-	2.97	8.91	23.31	6.015	11.29

*p<0,05 ,**<0,01 : Significant at levels of probability -

Table 3. Effect of micronutrients and on antioxidant properties of thyme in second harvest.

		Mean Square				
Sov	**df**	**Total phenols content (mg gallic acid/g DW)**	**Total flavonoids content (mg quercetin/g DW)**	**Nitric oxide radical scavenging activity (%)**	**DPPH radical scavenging activity (%)**	**Chain–breaking activity**
Block	2	2.91	0.005	25.87	18.53	37.04
Micronutrients	4	15.72	0.002	68.66**	163.1	44.21
Error	8	8.39	0.001	6.5	85.23	15.65
CV (%)	-	7.7	6.09	13.5	12.63	13.25

* p<0,05 , **<0,01: Significant at levels of probability

Total phenols content

In the trial phenolic content in the leaves of thyme ranged from 25.63 to 39.37 mg GAE/g DW in different micronutrient treatments. The highest total phenols content recorded in the Mn (39.37 mg Gallic acid/g DW) and the lowest was related to the control (28.9 mg Gallic acid/g DW) (Figure 1.).

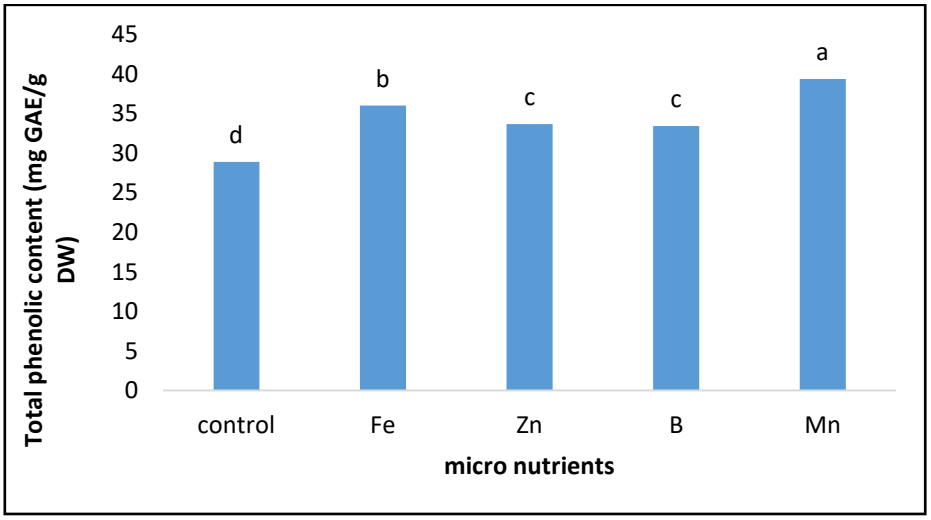

Figure 1. Mean comparison of total phenols content from first harvest of *Thymus vulgaris* L. affected by micronutrients

According to the results using micronutrients such as Fe, Zn, B, and Mn can lift phenolic content than control. In terms of this character the effect of micronutrients in the first harvest was significant ($P \leq 0.01$) whereas in second harvest there is not significant. Phenolic content in Mn treatment had significant difference than other treatments. The difference between Zn and B treatments was not significant. Yadegari (2015) indicated that foliar application of Mn, Fe and Zn increased total phenols content than control in *Borago officinalis.* Sabetsarvestani et al., (2013), reported that total phenolic contents in thyme were 19.65 and 19.06 mg GAE/g DW in top part and bottom part of shoots, respectively. According to some researches which done about antioxidant

activity, it is well–known that phenolic compounds contribute to nutritional value and quality in terms of modifying color, aroma, taste, and flavor and in providing health valuable impacts too (Vaya et al., 1997). The compounds are a class of antioxidant agents which act as free radical scavengers and are responsible for antioxidant activity in medicinal and aromatic plants (Shahidi and Wanasundara, 1992).

Total flavonoids content

The results showed that total flavonoids content in the leaves of the plant was significantly affected by micronutrients in first harvest ($P \leq 0.01$) whereas in second harvest there is not significant. (Table 2.). The highest total flavonoids content was recorded in the B treatment (1 mg quercetin/ g DW) and the lowest was related to the Fe treatment (0.509 mg quercetin/ g DW) (Figure 2.). Total flavonoids content in B treatment had significant difference than other treatments. The difference among control, Zn and Mn treatments were not significant. Yadegari (2015), showed that foliar application of B and Mn was significantly increased Total flavonoids content in plants of *Calendula officinalis* L. Ghandchi and Jamzad (2015), reported that total flavonoids contents of *Thymus trautvetteri* in different solvents were (2.076%, 1.468% and 1.412%) mg/g. Flavonoids as one of the most diverse and widespread group of natural compounds which there are in medicinal and aromatic plants possess a broad spectrum of chemical and biological activities. Flavonoids are a group of phytochemical compounds that there are widely in plants. And various biological activities of these compounds including the activities of antioxidant, antimicrobial, anti–inflammatory has been reported in many studies (Jamshidi et al., 2010). Kruma et al., (2008), reported that total flavonoids content in thyme were 0.376 mg/g in extracted with methanol.

Nitric oxide radical scavenging activity (%)

According to the results nitric oxide radical scavenging activity in the leaves of thyme was significantly affected by micronutrients in first and second harvest ($P \leq 0.01$) (Table 2.). In first harvest, the character ranged from 13.857% to 48.753%. The highest nitric oxide radical scavenging activity was recorded in Mn (48.753%) and the lowest was related to Fe (13.857%) (Figure 3.). In terms of this character, Mn treatment had significant difference than Fe and Zn but control and B same group. Using B and Mn lift the character whereas Fe and Zn decreased. In the second harvest, nitric oxide radical scavenging activity ranged from 13.847% to 26.593%.

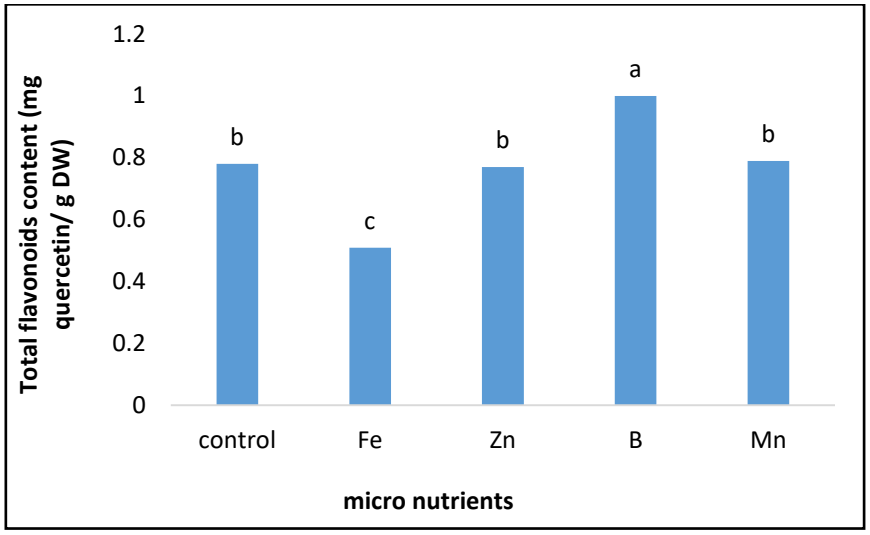

Figure 2. Mean comparison of total flavonoids content from first harvest of *Thymus vulgaris* L. affected by micronutrients

The highest was recorded in B (26.593%) and the lowest was related to Fe (13.847%) (Figure 4.). Tchamgoue et al., (2015), in a research about thyme indicated that nitric oxide scavenging activity in the seeds of the plant (extracted by methanol) ranged from 8.12% to 35.67%. Nitric oxide (NO) is a freely diffusible gaseous free radical. The interactions of NO with reactive oxygen species (ROS) such as H_2O_2 and O_2^- can be protective or cytotoxic (Beligni et al., 2002). At physiological level NO can limit oxidative injury but under high concentration of NO, a number of extremely reactive nitrogen

oxide species, such as N_2O_3 and $ONOO^-$ can be produced, which cause toxic reactions including lipid peroxidation, DNA modification and SH- oxidation. Natural extracts with nitric oxide scavenging ability prevent the toxic effects of excessive NO generation in the human body (Wink et al., 2001; Monkada et al., 1991).

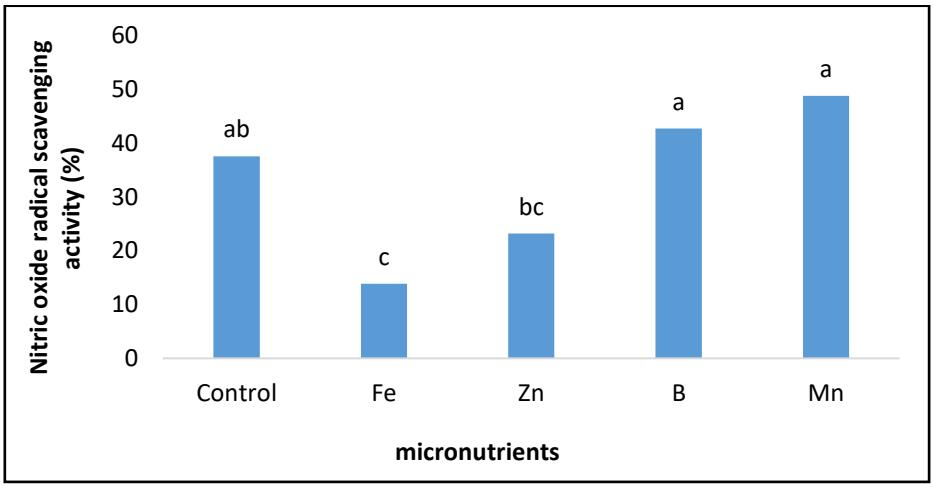

Figure 3. Mean comparison of nitric oxide radical scavenging activity (%) in first harvest of *Thymus vulgaris* L. affected by micronutrients

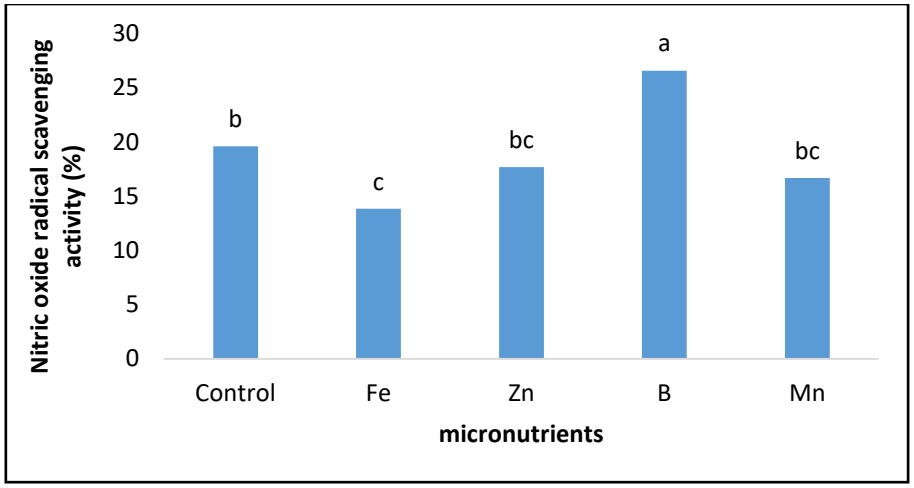

Figure 4. Mean comparison of nitric oxide radical scavenging activity (%) second harvest of *Thymus vulgaris* L. affected by micronutrients

DPPH radical scavenging activity (%)

The results indicated that DPPH radical scavenging activity (%) of leaves of thyme was significantly affected by micronutrients in first harvest ($P \leq 0.01$) whereas in second harvest there is not significant (Table 2.). DPPH radical scavenging activity in different treatments ranged from 68.4% to 91.7 %. The highest DPPH radical scavenging activity was recorded in control (91.7%) and the lowest related to Zn (68.4%) (Figure 5.). In terms of the character the control has significant difference with Fe and Mn whereas there is not with B and Zn. Farnad *et al*., (2014) reported that the highest antioxidant properties of peppermint (*Mentha piperita*) in methanol extract was 66.98 %. Free radicals may cause many disease conditions such as heart diseases and cancer (Javanmardi et al., 2003). The stable free radical DPPH° method is an easy, rapid, and sensitive way to survey the antioxidant activity of specific compounds or plant extracts (Ebrahimzadeh et al., 2008).

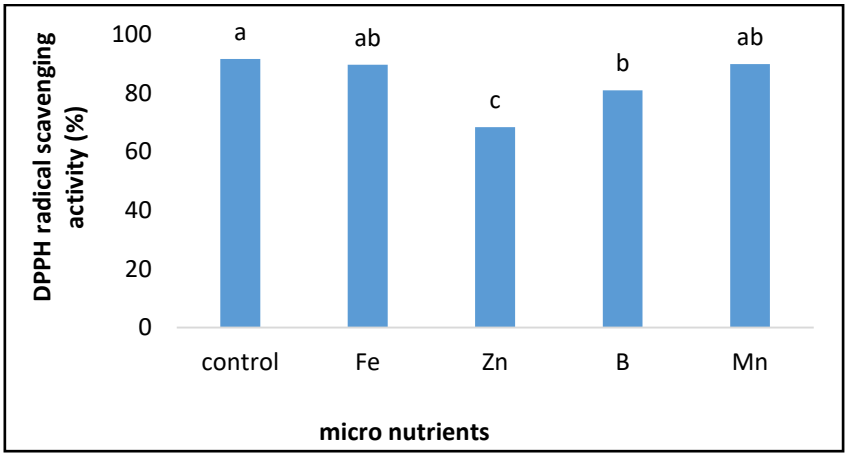

Figure 5. Mean comparison of DPPH radical scavenging activity (%) first harvest of *Thymus vulgaris* L. affected by micronutrients

Chain–breaking activity

The results showed that chain–breaking activity of the leaves of thyme was significantly affected by micronutrients in first harvest ($P \leq 0.01$) whereas there is not in second harvest (Table 2.). Chain–breaking activity in different treatments ranged from 43.81% to 65.41%. The highest percentage of the character was recorded in Mn (65.41 -Abs-3 /min/mg extract) and the lowest related to in Fe (43.81 -Abs-3 /min/mg extract) (Figure 6.). Chain–breaking activity in Mn treatment had significant difference than other treatments. The difference between Zn and Fe treatments was not significant.

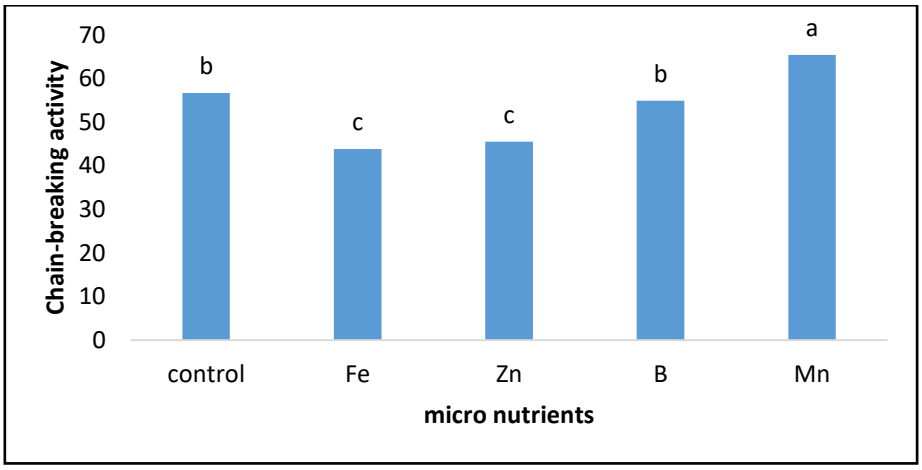

Figure 6. Mean comparison of Chain–breaking activity first harvest of *Thymus vulgaris* L. affected by micronutrients

Conclusions

The effect of micronutrients on antioxidant activity in the leaves of thyme generally is more effective in the first harvest than second. Application of micronutrients such as Fe, Zn, B, and Mn can lift phenolic content than control in the leaves of the plant moreover the effect of Mn is higher than others. In terms of this character the effective of these micronutrients is significant in first harvest. Using of B increase total phenolic content whereas Fe decrease in first harvest. Fe and Zn decrease Nitric oxide radical scavenging activity in first harvest; B increase the character in second harvest. Application of Zn and B decrease DPPH radical scavenging activity in first harvest. Using of Mn increase chain–breaking activity whereas Fe and Zn decrease in first harvest.

Acknowledgement: The authors acknowledge the help of Biological Department Laboratory of Urmia University, Urmia, West Azerbaijan province, Iran for help.

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