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Araştırma Makalesi/*Research Article (Original Paper)*

# The Effect of Micronutrients on Antioxidant Properties of Thyme (*Thymus vulgaris* L.) under Humic Acid Using Condition

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**Abstract:** The aim of the study was to investigate the special effects of micronutrients (Fe, Zn, B, and Mn) under humic acid using condition on Thyme antioxidant activity. The trial conducted at the Experimental Fields and laboratory of the Agronomy Department, Faculty of Agriculture, Urmia University, West Azerbaijan, Iran, during 2015-2016. Experiment was set in randomized complete block design and in three replications. In the trial the treatments were included 3 g Fe $L^{-1}$, 3 g Mn $L^{-1}$, 4 g B $L^{-1}$ and 2 g Zn $L^{-1}$ in each time and humic acid (300 kg per hectare) were given to all plots. Spraying of the elements was done at three times: Some antioxidant properties of thyme leaves such as total phenolic content, total flavonoid content, DPPH radical scavenging activity, nitric oxide radical scavenging activity and Chain-breaking activity were determined. The results of first harvest showed that the highest flavonoid (0.84 mg quercetin/ g dry weight) and nitric oxide radical scavenging activity (58.28 %) related to B. The highest total phenol content (31.92 mg Gallic acid/g dry weight), DPPH radical scavenging activity (92.5%) and chain-breaking activity (74.25-Abs-3 /min/mg extract) related to Mn, Control and Zn, respectively. The results of second harvest showed that the highest total phenol content (40.05 mg Gallic acid/g dry weight), flavonoid (0.65 mg quercetin/ g dry weight) and DPPH radical scavenging activity (87.36%) related to B + Humic acid, and the highest chain-breaking activity (37.43-Abs-3 /min/mg extract) related to control. In conclusion B has positive effect on increasing the activity of antioxidant of the plant than other treatments.

Keywords: Antioxidant Activity, Foliar Application, Humic acid, Micronutrients, Thyme

## Mikro Besin Maddelerinin Humik Asit Koşulları Altındaki Kekik (*Thymus vulgaris* L.)'in Antioksidan Özelliklerine Etkisi

**Öze**t: Bu çalışmanın amacı, mikro besin maddelerinin (Fe, Zn, B ve Mn) humik asit altındaki kekiğin antioksidan aktivitesi üzerine olan özel etkilerini araştırmaktır. Araştırma, 2015-2016 yılları arasında İran-Batı Azerbaycan, Urumiye Üniversitesi Ziraat Fakültesi Tarla Bitkileri Bölümü Deney Alanları ve Laboratuvarında gerçekleştirildi. Deneme tesadüfi blok deseninde ve üç tekerrürlü olarak yürütülmüştür. Araştırmada, her parsele her sulamada 3 gr Fe/L, Mn 3 gr Mn/L, 4 gr B/L, ve 2 gr Zn /L ve hümik asit (hektar başına 300 kg) olarak uygulanmıştır. Besin elementleri uygulamaları 3 defa yapılmıştır. Kekik yapraklarında toplam fenolik içeriği, toplam flavonoid içeriği, DPPH radikal giderimi aktivitesi, nitrik oksit radikal giderim aktivitesi ve Zincir kırma aktivitesi gibi bazı antioksidan özellikler belirlenmiştir. İlk hasatın sonuçları, B ile ilgili en yüksek flavonoid (0.84 mg quercetin / g kuru ağırlık) ve nitrik oksit radikal giderme aktivitesinin (% 58.28) en yüksek olduğunu ortaya koymuştur. Mn, Kontrol ve Zn ile ilgili sırasıyla en yüksek toplam fenol içeriği (31.92 mg Gallik asit / g kuru ağırlık), DPPH radikal süpürme aktivitesi (% 92.5) ve zincir kırma aktivitesi (74.25-Abs-3 / dak / mg ekstrakt) belirlenmiştir. İkinci hasatın sonuçları, B + Humik asit ile ilgili en yüksek toplam fenol içeriğinin (40.05 mg Gallik asit / g kuru ağırlık), flavonoidin (0.65 mg kerceretin / g kuru ağırlık) ve DPPH radikal temizleme etkinliğinin (% 87.36) en yüksek olduğunu ve kontrolle ilgili en yüksek zincir kırma aktivitesi (37.43-Abs-3 / dak / mg ekstrakt) olduğunu göstermiştir. Sonuç olarak, B, bitkinin antioksidan aktivitesini artırmak için diğer uygulamalara kıyasla olumlu etkiye sahip olmuştur.

**Anahtar kelimeler:** Antioksidan aktivite, Yaprak Uygulaması, Humik asit, Mikro besin maddeleri, Kekik

## Introduction

Physiological role of different antioxidants is to prevent injury to cellular components. In current period, a substantial form of indication developed supporting a main role for free radicals in many important reactions such as cellular reactions and suggesting that oxidative stress maybe be vital in the pathophysiology of some

communal diseases (Young and Woodside 2001). Each antioxidant compound is a substance while present in low concentrations associated to that of an oxidisable substrate, prevents the oxidation of the substrate (Halliwell and Gutteridge 1995). Vegetables and fruits have antioxidant compounds (polyphenols) which decrease oxidative stress in cells of plants and animals. Antioxidant characteristics of the compounds are answerable for their anticancer, antiviral, anti-inflammatory characters and etc. (Ninfali et al. 2005). Phenolic compounds as secondary metabolites extensively dispersed in different parts of plants. These compounds are significant components of numerous vegetables and fruits not only for their major impact on sensory potentials of them such as color, flavor, and taste, but also for their other properties such as antioxidant, anti-allergic, anti-mutagenic, and etc. (Alesiani et al. 2010). So, the doing of vegetables and fruits in prevention of some disease is partially related to the antioxidant characteristics of their essential phenolic compounds (Scalbert and Williamson 2000). Produced synthetic antioxidants in recent years are risky and unsafe; since of the toxicity these antioxidant and carcinogenicity, significant consideration directed to the identification of natural and safe antioxidants obtained from plants such as some medicinal and aromatic plants (Gordon 1996; Caia et al. 2004).

The *Thymus* as a genus which belongs to Lamiaceae family contains numerous species. Thyme (*Thymus vulgaris* L.) as a Mediterranean native species is small woody shrub. The plant is a medicinal and aromatic plant considered by broad chemical intraspecific (Figueiredo et al. 2008). The leaves of thyme used as a spice (fresh or dried) (Lee et al. 2004). The percentage of essential oil in thyme has been reported from 0.32% to 4.9% (Carlen et al. 2010). The plant essential oil shows several properties such as antibacterial, antifungal and antioxidant activities (Jackson and Hay 1994; Letchamo et al. 1995). The essences are rich in phenolics and possess a wide range of pharmacological and biological characteristics (Bozin et al. 2006), antimicrobial antiseptic, carminative, and antioxidative (Nejad et al. 2008).

According to some researches the major nonphenolic compounds are p-cymene and linalool (Atti-Santos et al. 2004; Goodner et al. 2006). Thymol and carvacrol, indicated to act as antioxidant (Jukic and Milos 2005). Cutillas et al. (2017), carried out a trial about eight samples of red thyme and winter thyme; they used several methods to evaluate antioxidant capacities in essential oil of the eight samples, concluding that their activities were mainly due to thymol and linalool. Some documents dedicated to the antimicrobial activity of the essence of thyme. Moreover, its antioxidant activity made its helpful for food safety (Amorati et al. 2013). Micronutrients have key effectiveness on growth and development of plants (Heidari et al. 2008). It is well accepted that micronutrients possess important role in photosynthesis, N-fixation, respiration and other metabolic processes in plants (Naga et al. 2013). Among micronutrients, Zinc (Zn) is a significant microelement related with some enzymatic activities such as photosynthetic system (Kizilgoz and Sakin 2010). Zinc is necessary in important enzymes and growth regulators (Datta et al. 2011). Iron (Fe) is essential in cytochrome structure; this element is one of the three micro vital elements for plants growth and production (Schonherr et al. 2005). Boron (B) made resistance of plasma membrane and combination by other minerals and essential for plant growth (Widom and Mihalkovic 2008). According to the result of some papers manganese (Mn) has most effective role for increasing oil production in oil plant (Nandi and Chatterjee 1991). The element acts such as cofactor for oxidases, dehydrogenases, and sugar transfer (Culotta et al. 2005). According to the results of a research, Fe and Zn have important role in essence biosynthesis in basil (*Ocimum sanctum* L.) (Misra et al. 2006). Rastghar (1998) showed that application of manganese as foliar increased yield and essential oil percentage. Nassiri and et al. (2010) showed that foliar application of iron and zinc increased yield of flower, essence content and yield. Humic acid is one of the widely used organic fertilizers. This organic fertilizer is made through the some chemical and biological decomposition of animal and plant matters by activities of microorganisms (Metzger 2010). Humic acid provides many benefits to crop production. The papers showed that it effects the growth of plants directly and indirectly. The direct act of humic acid on plant growth is as the cell chlorophyll content increasing, hormonal growth responses, the respiration process acceleration, in plant membranes increasing substances penetration, dry matter production changing, and nutrients uptake. Improvement of physical, chemical and biological conditions of soil is the indirect effects of humic acid. This organic material breaks up compacted soils and clay, enhances water retention, assists in transferring micro elements from the soil to the root of plants, increases the seeds germination, and stimulates the development of micro flora populations (Rao et al. 1987). In addition, humic acid possess constructive properties on the promotion of root growth of plants. Humic acid can lift the root/shoot ratio (Tattini et al. 1991).

There are no papers have been written about the effect of micronutrients on antioxidant activity of thyme under humic acid using condition. The chief goal of the submitted work was to investigate the effect of some micronutrients such as Fe, Zn, B, and Mn on antioxidant activity of thyme under Urmia condition, west Azerbayjan, Iran. The extracts of thyme leaves were investigated to define the total amount of phenol,

flavonoid, Chain-breaking activity (CBA), radical scavenging activity (DPPH) and Nitric oxide radical inhibition assay (NO°).

## Materials and Methods

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 showed.

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 | -1.8 | -22.8 | 16.4 | 2.0 |
| **February** | 33.2 | 0.1 | -22.0 | 21.0 | 2.5 |
| **March** | 51.5 | 5.3 | -19.0 | 26.0 | 3.3 |
| **April** | 61.3 | 11.0 | -12.0 | 30.8 | 4.0 |
| **May** | 44.3 | 15.7 | -1.6 | 31.8 | 3.5 |
| **June** | 14.2 | 20.3 | 4.0 | 36.2 | 3.4 |
| **July** | 5.5 | 23.9 | 9.8 | 38.0 | 3.1 |
| **August** | 2.4 | 23.5 | 8.0 | 39.2 | 3.0 |
| **September** | 4.7 | 19.3 | 2.2 | 36.0 | 3.0 |
| **October** | 24.3 | 13.4 | -5.0 | 30.0 | 2.6 |
| **November** | 39.6 | 6.8 | -13.4 | 23.0 | 2.2 |
| **December** | 28.6 | 1.3 | -20.0 | 21.4 | 2.0 |

* The government meteorological association of Iran

The trial was conducted at the experimental fields (37.53° N, 45.08° E, and 1320 m) of the Agronomy Department, Faculty of Agriculture and the Lab of Biology Department, Urmia University, Urmia, West azerbaijan, Iran, during 2015-2016, prepared in a randomized complete block design in three replications with plots of an area of 6 $m^2$. The land was plowed at the optimum moisture level (field capacity) and leveled. Phosphorus and Potassium fertilizers were applied at pre sowing time in autumn, according to soil analysis and farrowed in 50 cm. The seeds for sowing were obtained from Turkey. Sowing was done in a greenhouse 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. Humic acid (300 kg $ha^{-1}$) used synchronous with first irrigation in each plot of blocks. Foliar spraying of microelements included: control (water), Fe 3 g $L^{-1}$ water, Zn 2 g $L^{-1}$ water, B 4 g $L^{-1}$ water and Mn 3 g $L^{-1}$ water in each time. Spraying of the elements 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. Soil samples (0–30 cm) were taken in autumn before application of fertilizers. Soil analysis results of the soil samples in the field (Table 2) are shown.

*Extracts preparation*

Leaves of thyme harvested from the experimental field and were cut into minor pieces and dried (23 °C) and powdered. Methanol was the extraction solvents. Extraction procedure included the adding of 25 mL solvent to 2 g sample and shaking for 180 min at low speed. So the extract was passed through Whatman filter paper (Number 1.) (Whatman Ltd., England). Extraction was done using magnetic stirring. The solutions were sealed so kept at 4 °C until experiments. During the extraction process, light exposure avoided.

*Total phenolic content (TPC) determination*

Total phenolic contents of extracts measured with the Folin-Ciocalteu colorimetric method described previously (Kahkonen et al. 1999) using slight change. Folin Ciocalteu's phenol reagent (1 mL) and 10 % w/v

$Na_2CO_3$ (1 mL) added to sample extract (10 µl) and the mixture reaction incubated in the dark for 60 min. The absorbance of the reaction mixture measured at 750 nm. Total phenolic contents expressed in terms of g Gallic acid equivalents/ 100 g the plant leaves powder **(**The calibration equation for Gallic acid: y= 0.0415x- 0.0163).

Table2. Soil analyses results of the soil samples before sowing

| | | | |
|---|---|---|---|
| **EC** | 1.37 $dSm^{-1}$ | **O.C** | 1.38% |
| **$CaCO_3$** | 15.83% | **pH** | 7.8 |
| **B.S** | 47% | **K** | 315 mg kg-1 |
| **F.C** | 28% | **P** | 10.14 mg kg-1 |
| **Clay** | 43% | **Fe** | 18 mg kg-1 |
| **Loam** | 35% | **Zn** | 1.8 mg kg-1 |
| **Sand** | 22% | **B** | 0.3 mg kg-1 |
| **Texture** | Clay-Loam | **Mn** | 16 mg kg-1 |

*Total flavonoid content (TFC) determination*

Total flavonoid contents of extracts measured with aluminum chloride colorimetric method described previously (Youngjae et al. 2007) using slight change. 10 µl of extract diluted with 1 mL of water (deionized). Then 0.075 mL of 5 % $NaNO_2$ added to the mixture, which was allowed to stand for 5 minutes at room temperature (23 °C), and 0.15 mL of 10 % $AlCl_36H_2O$ added. The mixture was allowed to stand for 6 minutes at room temperature (23 °C), and 0.5 mL of 1 mol $L^{-1}$ NaOH added, and the total volume made up to 3 mL with water (deionized). The absorbance of the solution measured immediately at 510 nm. Total flavonoid content was expressed in terms of g quercetin equivalents/100 g thyme powder **(**The calibration equation for Gallic acid: y= 0.0772x- 0.0084).

*DPPH radical scavenging activity determination*

The free radical scavenging activity of the leaves of thyme extracts determined by a little change of the process described previously (Hatano et al. 1988). 10 µl of the extract added to a 2 mL of DPPH (1,1-diphenyl 2-picryl hydrazyl). The solution incubated for 30 minutes in the dark condition at room temperature (23 °C). After the incubation, the mixture absorbance estimated at 517 nm. The DPPH radical scavenging activity property calculated according to the following formula:
Percentage inhibition: [(A blank – A sample) / A blank] × 100

*Nitric oxide radical scavenging activity determination*

Nitric oxide radical inhibition measured using Griess Ilosvay reaction (Garrat 1964). In this examination, Griess Ilosvay reagent is changed by applying 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) incubated at 25 °C for 150 minutes. After incubation, 0.5 mL of the reaction mixture mixed with 1 mL of sulfanilic acid reagent (0.33 % in 20 % glacial acetic acid) and allowed to stand for 5 minutes to complete diazotization. Before, 1 mL of naphthyl ethylene diamine dihydrochloride added, mixed and allowed to stand for 30 minutes at 25 °C. A pink colored chromophore formed in diffused light. Gallic acid and ascorbic acid used such as positive controls. The absorbance of these solutions estimated at 540 nm against the corresponding blank solutions. The nitric oxide radical scavenging activity 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 process of Brand-Williams et al. (1995) with a little change. The Chain-breaking activity 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 expressed as k. Antioxidant activity reported as ($-Abs^{-3}$ /min/mg extract).

*Statistical analysis*

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.

## Results and Discussion

The results showed that the impact of treatments in the first harvest on total phenols content ($P \leq 0.01$), total flavonoids content ($P \leq 0.01$), nitric oxide radical scavenging ($P \leq 0.01$), DPPH radical scavenging activity ($P \leq 0.05$) and Chain-breaking activity ($P \leq 0.01$) was significant. In second harvest, the impact of micronutrients on total phenols content ($P \leq 0.05$), total flavonoids content ($P \leq 0.01$), DPPH radical scavenging activity ($P \leq 0.05$) and Chain-breaking activity ($P \leq 0.01$) was significant.

Table 2- Effect of micronutrients 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 | 15.87 ** | 0.0006 | 37.06 | 1.27 | 0.045 |
| Treatments | 4 | 16.3 ** | 0.117 ** | 903.2** | 149.97 * | 1925.7 ** |
| Error | 8 | 0.33 | 0.005 | 49.2 | 24.19 | 58.92 |
| Cv (%) | - | 1.96 | 11.37 | 25.04 | 5.91 | 17.25 |

* and ** : Significant at levels of probability 5% and 1%

Table 3- Effect of micronutrients 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 | 9.49 | 0.008 * | 6.59 | 14.02 | 4.16 |
| Treatments | 4 | 52.52 * | 0.012 ** | $41.32^{ns}$ | 530.86 * | 294.93 ** |
| Error | 8 | 9.02 | 0.001 | 132.45 | 115.41 | 37.33 |
| Cv (%) | - | 8.44 | 6.69 | 23.42 | 14.07 | 26.43 |

* and ** : Significant at levels of probability 5% and 1%

Table 4- Effect of micronutrients on antioxidant properties of thyme in first harvest

| | Mean comparison | | | | |
|---|---|---|---|---|---|
| Treatments | 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 |
| Control+ humic acid | 29.67 bc | 0.5 b | 13.82 b | 92.5 a | 59.6 b |
| Fe + humic acid | 25.69 d | 0.36 c | 22.32 b | 81.63 bc | 50.33 b |
| Zn + humic acid | 29.47 c | 0.75 a | 22.43 b | 81.49 bc | 74.25 a |
| B + humic acid | 30.65 b | 0.84 a | 58.283 a | 73.43 c | 11.007 d |
| Mn + humic acid | 31.92 a | 0.74 a | 23.203 b | 86.76 ab | 27.22 c |

Means, in each column, followed by similar letter(s) are not significantly different

Table 5- Effect of micronutrients on antioxidant properties of thyme in second harvest

| | Mean comparison | | | | |
|---|---|---|---|---|---|
| Treatments | 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 |
| Control+ humic acid | 29.744 c | 0.53 bc | 19.54 a | 74.17 ab | 37.437 a |
| Fe + humic acid | 36.331 ab | 0.62 a | 25.47 a | 54.43 b | 18.117 bc |
| Zn + humic acid | 33.101 bc | 0.6 ab | 21.86 a | 85.707 a | 29.333 ab |
| B + humic acid | 40.055 a | 0.651 a | 23.42 a | 87.36 a | 14.01 c |
| Mn + humic acid | 38.62 ab | 0.49 c | 31.17 a | 80. 06 a | 16.667 c |

Means, in each column, followed by similar letter(s) are not significantly different

*Total phenols content*

Numerous thousand molecules having a polyphenol structure have been identified in higher plants (Jayasingne et al. 2003). It is usually ascribed to the presence of polyphenols, since high contents of phenolic compounds are usually connected with potent antioxidant properties. It is illustrious that the compounds contribute to quality and nutritional value in terms of changing color, taste, aroma, and flavor (Vaya et al. 1997). Phenolic compounds in the leaves of *Thymus vulgaris*, as determined by the Folin-Ciocalteu method. The effects of treatments on total phenols content were significant in the first and second harvest. The highest total phenols content in first harvest was recorded in the Mn + Humic acid (31.92 mg Gallic acid/g dry weight) and the lowest was related to the Fe + Humic acid (25.69 mg Gallic acid/g dry weight) (Figure 1).The results of second harvest showed that the highest total phenols content (40.05 mg Gallic acid/g dry weight) was recorded in B + Humic acid. And the lowest was related to the Control + Humic acid (29.74 mg Gallic acid/g dry weight). Between Mn + Humic acid and Fe + Humic acid treatments was no significant difference, and this treatment had the second rank (Figure 2). 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. In thyme the main phenolic compounds are glycuronids of apigenin, luteolin, eriodyctiol, luteolin glycosides, rosmarinic acid, quercitine (Justesen 2000; Guillen and Manzanos 1998). Kruma et al. (2008) reported that total phenolic compounds in latvian thyme were 74.96 mg/g. Sadowska et al. (2017) evaluate the effects of three drying methods (under natural conditions, at temperature of 35 °C/40 °C, and freeze-dried) of thyme on some biochemical properties. According their result, the highest content of polyphenols for thyme was found at 35 °C drying. Asensio-S.-Manzanera et al. (2011) indicated that total phenols content in ten populations of *Thymus mastichina* collected from different origins of Spain in 2009, ranged from 10.27 to 23.60 (mg GAE/g). Amzad Hossain et al. (2013) indicated that among the five crude extracts, butanol extract contained the highest (245.26 mg/g) amount of phenol compounds followed by hexane extract (160.35 mg/g), chloroform extract (158.5 mg/g), ethyl acetate extract (84.85 mg/g), and methanol (49.43 mg/g). Medicinal and aromatic plants are the best sources for chemical ingredients, antimicrobial and antioxidant agents for cure of different diseases. Without paying attention to the effect of micronutrients crude extracts from the plant showed good amounts of total phenol. Nickavar and Esbati (2016), reported that total phenolic content were 295.93, 337.00, and 295.57 (µg rutin/mg extract) three Thymus species, *T.daenensis, T. kotchyanus, and T. pubescens* respectively. Tohidi *et al*., (2017) in a trial about nine Thymus species from different regions of Iran reported that total phenolic in *T. migricus, T. fallax, T. serpyllum, T. trautvetteri, T. transcaspicus, T. carmanicus, T. fedtschenkoi, T. daenensis, and T. pubescence recorded* were 37.75, 57.38, 67.8, 53.52, 65.76, 37.58, 52.76, 70.56, 31.38, 34.37, 44.77, 43.67, 50.33, 62.40 (mg TAE/ g DW) respectively.

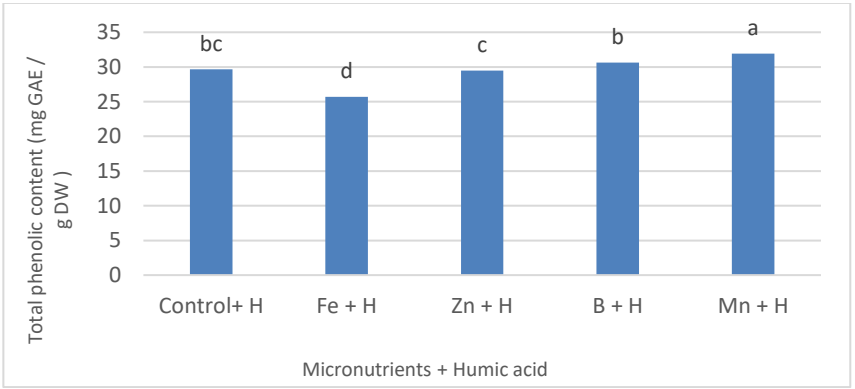


Figure 1. Mean comparison of total phenols content from first harvest of *Thymus vulgaris* L. affected by micronutrients and humic acid.

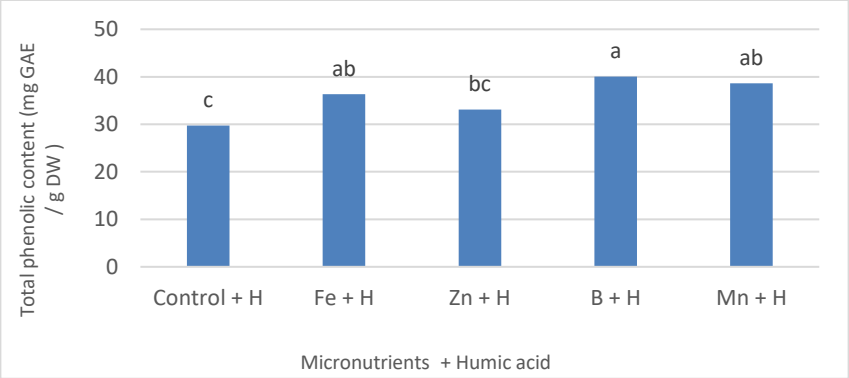


Figure 2. Mean comparison of total phenols content from second harvest of *Thymus vulgaris* L. affected by micronutrients and humic acid.

*Total flavonoids content*

Flavonoids as one of the most diverse and widespread group of natural compounds, have a broad spectrum of chemical and biological activities. The results showed that total flavonoids content of the thymus vulgaris were significantly affected by treatments in both harvest. (Table 2 and 3). The highest total flavonoids content in first harvest was recorded in the B + Humic acid (0.84 mg quercetin/ g dry weight) and the lowest was related to the Fe + Humic acid (0.36 mg quercetin/ g dry weight) (Figure 3). The highest total flavonoids content in second harvest was recorded in B + Humic acid (0.651 mg quercetin/ g dry weight) and the lowest was related to the Mn + Humic acid (0.49 mg quercetin/ g dry weight) (Figure 4). Ghandchi and Jamzad (2015), reported that total flavonoids contents of Thymus trautvetteri in different solvents were (2.076%, 1.468% and 1.412%) mg $g^{-1}$. Kruma et al. (2008) reported that total flavonoids content in thyme were 0.376 mg/g in extracted with methanol. Amzad Hossain et al. (2013) reported that among the five crude extracts of *Thymus vulgaris*, methanol extract contained the highest (1.71 mg $g^{-1}$) amount of flavonoids content compounds followed by butanol (1.55 mg/g), chloroform (1.37 mg $g^{-1}$), ethyl acetate (1.29 mg/g) and hexane (1.18 mg $g^{-1}$). Miura and Nakatani (1989), using acetone as the solvent after removing the non-polar components of the leaves of T. vulgaris L., and following a complex fractionating scheme, found six flavones, one of them not detected before by the authors mentioned above, namely: 5-hydroxy-7,4'-dimethoxyflavone (7,4-dimethylapigenin, MW = 298) not found here. Several authors already reported on flavonoids groups exhibited a wide range of biological activities such as antioxidant, anti-inflammatory, antimicrobial, anti-angionic, anticancer and anti-alergic (Amzad Hossain et al. 2013). Nickavar and Esbati (2016), in a trial indicated that flavonoid content were 35.21, 37.11, and 50.39 (µg rutin/mg extract) in three Thymus species, *T.daenensis, T. kotchyanus, and T. pubescens* respectively. Tohidi et al. (2017) indicated that flavonoid contects in *T. migricus, T. fallax, T. serpyllum, T. trautvetteri, T. transcaspicus, T. carmanicus, T. fedtschenkoi, T. daenensis, and T. pubescence* were 4.26, 8.14, 1.89, 4.28, 4.93, 4.16, 3.94, 2.5, 6.34, 3.07, 8.01, 3.04, 1.98, 8.55 (mg QE/ g DW) respectively.

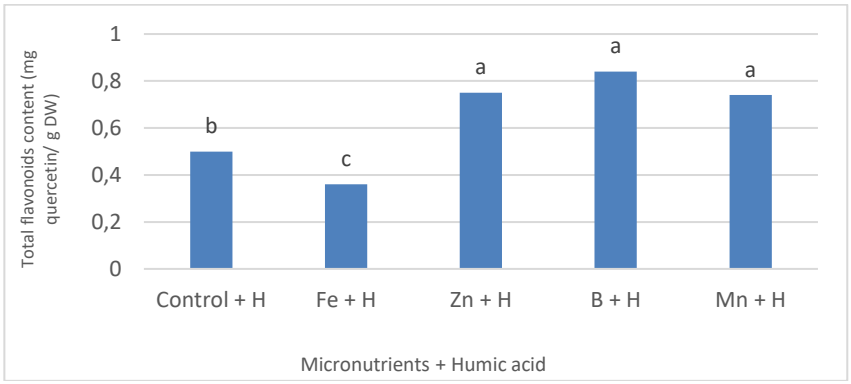


Figure 3. Mean comparison of total flavonoids content from first harvest of *Thymus vulgaris* L. affected by micronutrients and humic acid.

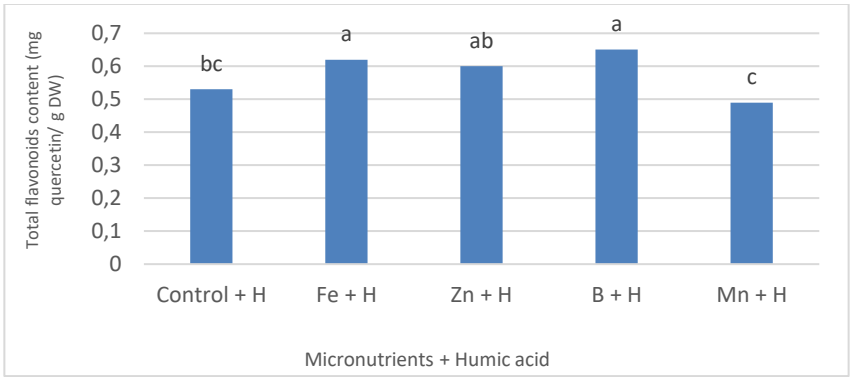


Figure 4. Mean comparison of total flavonoids content from second harvest of *Thymus vulgaris* L. affected by micronutrients and humic acid.

*Nitric oxide radical scavenging activity (%)*

Nitric oxide radical inhibition can be estimated by the use of Griess Ilosvay reaction (Garrat 1964). The effects of treatments on Nitric oxide radical scavenging activity were significant in the first harvest (Table 2). The highest Nitric oxide radical scavenging activity (%) was recorded in the B + Humic acid (58.28 %) and the lowest was related to Control + Humic acid (13.82 %) (Table 4). The effects of treatments on Nitric oxide radical scavenging activity was not significant in the secont harvest (Figure 5). Parul et al. (2012) reported that nitric oxide radical scavenging activity in leaves of triumfetta rhomboidae by the methanolic extracts were 20.294 to 53.942% in different concentrations. The chemical constituents in the plants or crude extracts are known to be biologically active ingredients. Some chemical constituents are considered as secondary metabolites components. They are directly responsible for different activity such as antioxidant, antimicrobial, antifungal and anticancer (Hossain and Nagooru 2011; Harborne 1998; Kokate 1997). All these secondary metabolites components showed antioxidant and antimicrobial properties through different mechanism (Hossain et al. 2011).

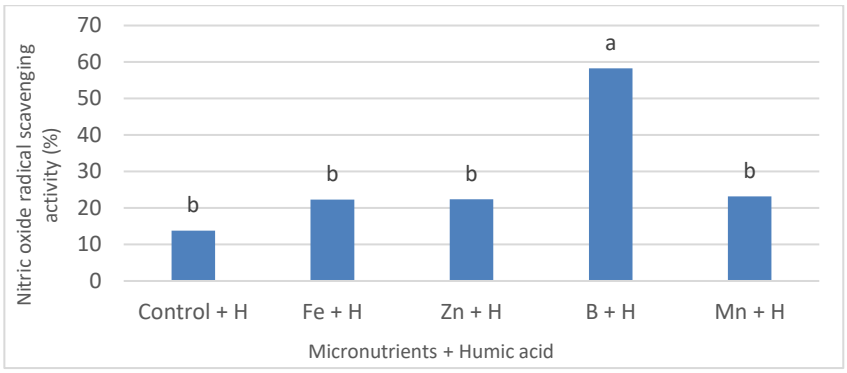


Figure 5. Mean comparison of nitric oxide radical scavenging activity (%) in first harvest of *Thymus vulgaris* L. affected by micronutrients and humic acid.

*DPPH radical scavenging activity (%)*

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). Antioxidant activity is most commonly evaluated by the DPPH scavenging activity test (Baharfar et al. 2015). The results showed that DPPH radical scavenging activity (%) of the thymus vulgaris were significantly affected by treatments in both harvest. The highest DPPH radical scavenging activity (92.5%) in first harvest was recorded in the control + Humic acid, and the lowest was related to the B + Humic acid (73.43%) (Figure 6). The highest DPPH radical scavenging activity (87.36 %) in second harvest was recorded in B + Humic acid. the lowest (54.43 %) was related to Fe + Humic acid (Figure 7). *Istrati et al.* (2013) reported that DPPH radical scavenging activity (%) in thymus vulgaris were 75.86 ± 0.62 in extracted with water. Asensio-S.-Manzanera et al. (2011) indicated that DPPH in ten populations of *Thymus mastichina* collected from different origins of Spain in 2009, ranged from 1.78 to 0.59 (EC50 mg $mL^{-1}$). Aazza et al. (2011) in a research on some aromatic plants investigated on their antioxidant activity; according to their results DPPH in Thymus vulgaris was 0.259 (EC50 mg $mL^{-1}$).

According to the results all the samples from different micronutrients treatments possessed the potent free radical scavenging and antioxidant activities in different assays.

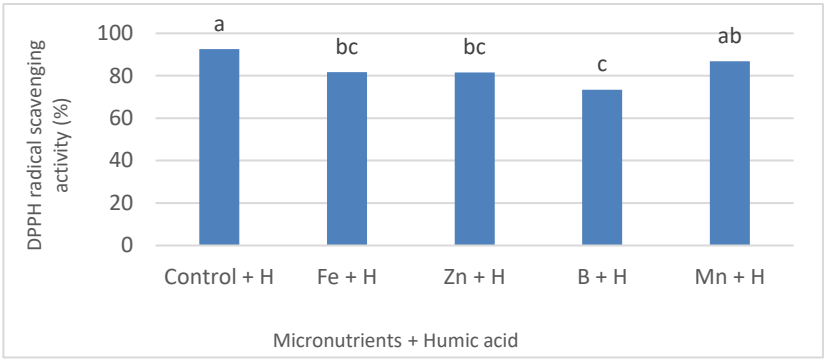


Figure 6. Mean comparison of DPPH radical scavenging activity (%) first harvest of *Thymus vulgaris* L. affected by micronutrients and humic acid.

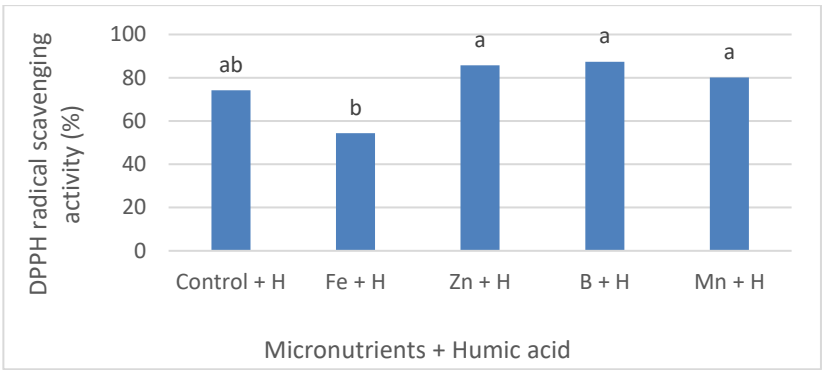


Figure 7. Mean comparison of DPPH radical scavenging activity (%) second harvest of *Thymus vulgaris* L. affected by micronutrients and humic acid.

*Chain-breaking activity*

Antioxidants are essential substances able to protect the human body from deleterious effects of oxidative stress. A common feature of the plants is their antioxidant activity. The results showed that Chain-breaking activity of the thymus vulgaris were significantly affected by treatments (Table 2 and 3). The highest Chain-breaking activity in first harvest was seen in the Zn + Humic acid (74.25 -Abs-3 /min/mg extract). And the lowest Chain-breaking activity was observed in B + Humic acid (11.007 -Abs-3 /min/mg extract) (Figure 8). The highest Chain-breaking activity (37.43%) in second harvest was observed in Control + Humic acid, and the lowest Chain-breaking activity (14.01%) was observed in B (Figure 9). Without considering micronutrients use in the trial, the leaves with strong antioxidant activities help protect cells against the oxidative damages caused by free-radicals. They may be used to differentiate potent chain-breaking antioxidants and compounds propagating radical chain reactions.

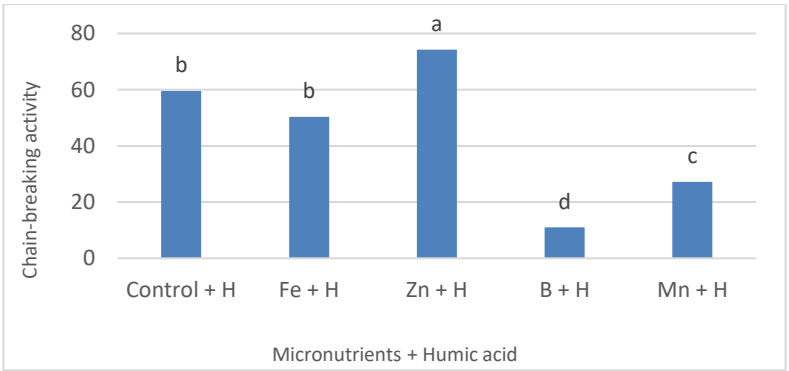


Figure 8. Mean comparison of Chain-breaking activity first harvest of *Thymus vulgaris* L. affected by micronutrients and humic acid.

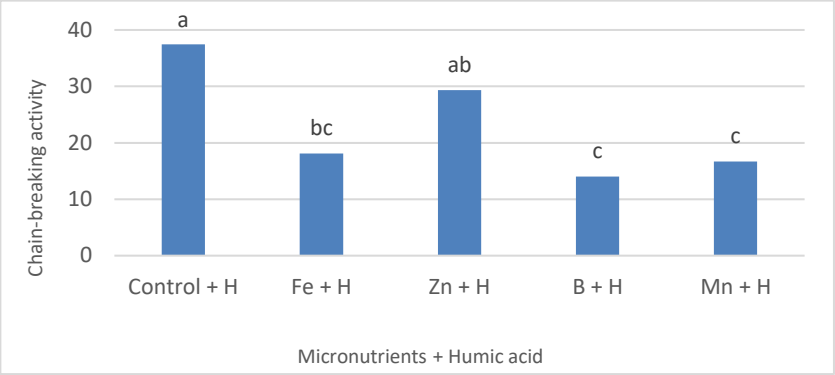


Figure 9. Mean comparison of Chain-breaking activity second harvest of *Thymus vulgaris* L. affected by micronutrients and humic acid.

## Conclusions

The knowledge emerging from the results may be useful in terms of antioxidant activity for the cultivation and using of manures in thyme farms. The results in first harvest showed that the highest total flavonoids content and nitric oxide radical scavenging activity was observed in B + Humic acid. The highest total phenols content, DPPH radical scavenging activity and the Chain-breaking activity was observed in Mn + Humic acid, Control + Humic acid and Zn + Humic acid, respectively. The results in second harvest showed that the highest total phenols content, total flavonoids content and DPPH radical scavenging activity was observed in B + Humic acid and the highest Chain-breaking activity was observed in Control + Humic acid. According to the results, B + Humic were the best. Future study may be directed toward the investigation of the compositions of essential oil, enzymes or genes underlying the biosynthetic pathways under different nutrient treatments in thyme producing to provide new insights into the possibilities for increasing the major compounds in the plant.

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