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Evaluation of Antioxidant Activity of Garden Thyme (*Thymus vulgaris* L.) Affected by Humic Acid Under Urmia-Iran Condition

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Abstract: The trial aimed to study the effects of different levels of humic acid on the antioxidant activity of garden thyme. The study was conducted at the experimental fields of the Agronomy Department, Faculty of Agriculture, Urmia University, West Azerbaijan, Iran, and used randomized complete block design with four replications. Three different humic acid doses; 200 kg ha^{-1}, 400 kg ha^{-1} and 600 kg ha^{-1}, and control with no humic acid were the treatments. Total phenolic content, total flavonoid content, DPPH (1,1-diphenyl 2-picryl hydroxyl) radical scavenging activity, nitric oxide radical scavenging activity, and chain-breaking activity were determined. According to the results, the effect of different levels of humic acid in the first harvest on total flavonoid content and nitric oxide radical scavenging was significant whereas, in the second harvest, total phenols content, total flavonoids content, DPPH radical scavenging activity, and chain-breaking activity were significant. The maximum total phenolic content, and nitric oxide radical scavenging activity were obtained in the control treatment. The highest total flavonoid content, DPPH radical scavenging activity, and chain-breaking activity were obtained in the application of 400 kg ha^{-1} and 600 kg ha^{-1} humic acid.

Keywords: Medicinal-aromatic plant**,** antioxidant activity, garden thyme, humic acid

1. Introduction

In the body, harmful oxidative stress happens when the production of free radicals and active intermediates surpasses the system's ability to thwart (Rahman, 2013). The free radicals interact with other molecules, they cause oxidative damage that can increase a variety of diseases. A substantial body of indication developed supporting a key role for free radicals in many fundamental cellular reactions and suggesting that oxidative stress might be important in the pathophysiology of common diseases such as chronic renal failure, atherosclerosis, and diabetes mellitus (Young and Woodside, 2001). During endogenous metabolic reactions, aerobic cells produce reactive oxygen species such as superoxide anion, hydrogen peroxide, hydroxyl radical, and organic peroxides. The transfer of an electron to molecular oxygen takes place at the level of the respiratory chain, and the electron transport chains are located in membranes of the mitochondria. Proteins and lipids are significant targets for oxidative attack too, and formation of these molecules can increase the risk of mutagenesis in the body. The skin is exposed to the environment naturally, which is necessary to protect against oxidizing species. Acting to protect the organism against these harmful pro-oxidants is a complex system of enzymatic antioxidants and non-enzymatic antioxidants, vitamins C and D (Reuter et al., 2010).

An effective antioxidant material significantly delays or inhibits the oxidation property of a substrate (Halliwell and Gutteridge, 1995). The studies of antioxidants concern both sources of antioxidants, natural and synthetic (Augustyniak et al., 2010). In the last century, butylated hydroxyl

anisole has been used as an antioxidant in foods. However, the use of this synthetic molecule has been associated with possible toxicity, and it has been reported that it has some side effects such as carcinogenesis, which has led to some restraint in its use (Haigh, 1986). Natural extracts from plant origin could provide alternatives to synthetic preservers, namely antioxidants, also providing bioactive properties and bringing additional value to the final products (Attia et al., 2017). The search, characterization, and application of natural antioxidants remain in the focus of numerous research teams all over the world. Therefore, the scope of information in this area is extremely large, diverse, and rather difficult for systematic reviewing and assessment. The interest in natural antioxidants is determined by the universality of their action in various redox systems and consequently broad spectra of possible applications. Anti-oxidative phytochemicals are considered functional ingredients for pharmaceuticals, functional foods, dietary supplements, animal feed, cosmetics, and other products (Augustyniak et al., 2010).

Medicinal and aromatic plants contain antioxidant compounds called polyphenols that are recognized to thwart oxidative stress in the body of organisms (Ninfali et al., 2005). Phenolic compounds are secondary metabolites, widely distributed in plants and their different pharmacological effects have been recognized (Alesiani et al., 2010). Some bakery, dairy, and meat products have already been developed incorporating natural extracts from aromatic plants, spices, and fruit powder, for antioxidant purposes. In particular, aqueous extracts were prepared from *Foeniculum vulgare* Mill. (fennel) and *Matricaria recutita* L. (chamomile) were successively incorporated as natural antioxidants and antimicrobials for cottage cheese (Caleja et al., 2017). The genus *Thymus*, predominantly found in the Mediterranean region, Asia, Southern Europe, and North Africa, is constituted by more than three hundred species. There are several ecotypes, which differ in their morphological characteristics and the composition of their essential oils, although all of them are characterized by a moderate odor and sometimes a very pronounced balsamic and spicy flavor (Cutillas et al., 2018). *Thymes* are of increasing economic importance in North America, Europe and North Africa (Letchamo and Gosselin, 1996). Many ethnomedicinal properties are attributed to infusions, decoctions, and essential oils of the aerial parts of Thymus species, which are used due to their tonic, carminative, digestive, antispasmodic, antimicrobial, antioxidant, antiviral, anti-inflammatory, and expectorant properties, as well as for the treatment of colds (Nickavar et al., 2005; Pirbalouti et al., 2013). Garden thyme (*Thymus vulgaris* L.) a member of the family Lamiaceae is grown in different areas of the Mediterranean and Asia. Garden *thyme* has been used medicinally since ancient times (Hornok, 1992). Aromatic plants and their essential oils are increasingly studied for use in the chemical, cosmetic, food, fragrance, and pharmaceutical industries due to their potential bioactivities. This is particularly the case with the essential oils from *Thymus* species due to the presence of bioactive compounds. Indeed, *thyme* essential oil is among the world's ten most commonly used essential oils as a food preservative. They may diminish oxidative processes in food and cosmetic products and so be used to replace synthetic antioxidants, increasing consumer acceptance of the products. In the same way, they have a potential for use in human health care, since, they may reduce the oxidative stress that often enhances disease development (Cutillas et al., 2018).

Humic acid is extracted from different sources such as soil, humus, peat, oxidized lignite, and coal. Humic acid can directly have positive effects on plant growth and increase the growth of shoots and roots, absorption of nitrogen, potassium, calcium, magnesium, and phosphorus by the plant. Humic acid is consistent with nature and is not dangerous for the plant and environment (Haghighi et al., 2011). AbdelMawgoud et al. (2007) state that humic acid increases plant growth through chelating different nutrients to overcome the lack of nutrients, and has useful effects on growth increase, production, and quality improvement of agricultural products due to having hormonal compounds. It has been reported that the application of humic acid increases plant growth and absorption of nutrients (Amini et al., 2019; Özyazıcı, 2020; Aslan and Sarıhan, 2021). There are a few papers that have been written about the effect of humic acid on the antioxidant activity of garden thyme. The chief goal of the submitted work was to investigate the effect of different amounts of humic acid on the antioxidant activity of garden thyme (*T. vulgaris* L.) under Urmia conditions, West Azerbaijan, Iran.

2. Materials and Methods

2.1. Research area and growth conditions

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 m above sea level) at Urmia University, Iran, in 2016. The study was conducted according to a randomized complete blocks design with four replications and 6 m^2 plots.

West Azerbaijan province is located at the utmost end of Iran's northwest, between 35 degrees 58 minutes and 46 degrees North Latitude, and also between 44 degrees 3 minutes and 47 degrees 23 minutes longitude. The province has an area of 37614 km^2 (Rahimi et al., 2019). The main climatic properties of the studied site were shown in Table 1.

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

Months	Rainfall (mm)	Average temperature (°C)	Minimum temperature (°C)	Maximum temperature (°C)	Wind speed (m s^{-1})
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
Total/Mean	338.9	11.6	-7.7	29.2	2.9

*: The government meteorological association of Iran

Soil samples (0-30 cm) were taken in autumn before the application of fertilizers. Soil analysis results of the experimental soil samples in the field (Table 2) are shown. The soils were clay loam textured, salt-free, slightly alkaline and had more lime content, semi-low organic matter, moderate available phosphorus, and high available potassium content (Table 2).

Table 2. Soil analyses results of the experimental soil samples in the field before seedling sowing (0-30 cm)

Soil properties	Unit	Value
Clay	%	44
Loam	%	34
Sand	%	22
pH		7.7
Organic matter	%	1.28
Electrical conductivity	dS m^{-1}	1.32
$CaCO_3$	%	16.3
Total nitrogen	%	0.096
Available phosphorus	mg kg^{-1}	9.54
Available potassium	mg kg^{-1}	320
Available iron	mg kg^{-1}	17
Available zinc	mg kg^{-1}	1.6
Available boron	mg kg^{-1}	0.4
Available manganese	mg kg^{-1}	15

The seeds for sowing were obtained from Turkey. Sowing was carried out in a greenhouse at the Department of Horticulture, Faculty of Agriculture, Urmia University, during the period from 21. 03. 2016 till 06.05.2016. The seeds were sown in plastic pots filled with soil, sand, and peat moss substrate as a material for germination.

The land was plowed at the optimum moisture level (field capacity) and leveled. According to soil analysis (Table 2), nitrogen and phosphorus fertilizers were applied at pre-sowing in the autumn. The amount of nitrogen and phosphorus used in the soil was 120 and 80 kg ha^{-1}, respectively. After planting, irrigation was performed according to weather conditions and plant growth stage. Seedlings were transferred to the experimental field.

Application of humic acid included: control, 200 kg ha^{-1}, 400 kg ha^{-1} and 600 kg ha^{-1} humic acid doses. Humic acid was applied between planting rows and then mixed with the soil in autumn 2016. Harvestings were done in 50% flowering in the second year two times.

2.2. Phytochemical analysis

Fresh leaves of thyme were cut into small pieces and dried and powdered at room temperature in shade at first. The methanolic extraction was as the addition of 25 mL solvent to 2 g sample and was shaken for 60 min at 1000 rpm and then the extract was passed through Whatman filter paper No.1 (Whatman Ltd., England). The solutions were then stored at 4 °C until experiments. Light exposure was avoided during the extraction process (Farnad et al., 2014).

2.3. Total phenolics contents

Total phenolics contents (TPC) of extracts were estimated with the Folin-Ciocalteu colorimetric

method described previously (Kulisic et al., 2005). Folin Ciocalteu's phenol reagent (1 mL) and 10% w/v Na_2CO_3 (1 mL) were added to 10 µL sample extract 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 was expressed in terms of g gallic acid equivalents $100g^{-1}$ *Thymus vulgaris* powder (The calibration equation for gallic acid: y= 0.0415x- 0.0163).

2.4. Total flavonoids content

Total flavonoid contents (TFC) of extracts were estimated with the aluminum chloride colorimetric method based on Gallego et al. (2013). 10 µL of the extract was diluted with 1 mL of deionized water. Then 0.075 mL of 5% $NaNO_2$ was added to this mixture, which was allowed to stand for 5 min at room temperature, and 0.15 mL of 10% $AlCl_3.6H_2O$ was added. The mixture was allowed to stand for 6 min at room temperature, 0.5 mL of 1 mol L^{-1} 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 was expressed in terms of g quercetin equivalents $100g^{-1}$ garden thyme powder (The calibration equation for gallic acid: y= 0.0772x- 0.0084).

2.5. DPPH radical scavenging activity

The free radical scavenging activity of plant extracts was determined according to Youdim et al. (2002). Accordingly, 10 µL of the extract was added to a 2 mL of DPPH (1,1-diphenyl 2-picryl hydroxyl). 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 Equation 1 (Rahimi et al., 2019).

$$\text{Inhibition}\,(\%) = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (1)$$

Where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the sample, respectively.

2.6. Nitric oxide radical scavenging activity

Nitric oxide radical inhibition can be estimated by the use of the Griess Ilosvay reaction (Jukić and Miloš, 2005). In this investigation, the Griess Ilosvay reagent is modified by using naphthyl ethylene diamine dihydrochloride (0.1% w v^{-1}) instead of 1-naphthylamine (5%). The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (0.5 mL), and thyme leaf 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 ethylenediamine 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 (NORSA) was calculated according to Equation 2 (Rahimi et al., 2019).

$$\%\ \text{NORSA} = [(A_{blank} - A_{sample}) \times 100]/A_{sample} \quad (2)$$

Where A_{blank} is the absorbance of the control and A_{sample} is the absorbance of the sample, respectively.

2.7. Chain-breaking activity

The chain-breaking activity (CBA) was based on the method of Taghipour et al. (2017) with slight modification. The chain-breaking activity was expressed by the reaction rate k and calculated by the following Equation 3 (Rahman, 2013).

$$\text{CBA}\,(\%): A_{bs}^{-3} - A_{bs0}^{-3} = -3kt \quad (3)$$

Where A_{bs0} is initial absorbance, A_{bs} is the absorbance at the increasing time, (t), and the reaction rate was expressed as k. Antioxidant activity was reported as ($-Abs^{-3}$ /min/mg extract).

2.8. 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 the LSD (Least Significant Difference) test. The results are given as mean ±standard deviation in the tables.

3. Results and Discussion

The results showed that the effect of different levels of humic acid in the first harvest on total flavonoids content and nitric oxide radical scavenging was significant ($p<0.01$). And in the second harvest, the effect of different levels of humic acid on total phenols content, total flavonoids content, DPPH radical scavenging activity, and chain-breaking activity were significant.

3.1. Total phenols content

In terms of TPC, the effect of different levels of humic acid in the second harvest was significant ($p<0.01$) whereas in the first harvest it was not significant. Total phenol content was ranged from 28.71±1.22 to 29.34±1.61 mg gallic acid g DW^{-1} at

the first harvest (Table 3). The highest TPC was recorded in the control (35.38±1.81 mg gallic acid g DW^{-1}) and the lowest was in the 400 kg ha^{-1} (26.21±1.50 mg gallic acid g DW^{-1}) (Table 4). According to the results, using different levels of humic acid reduced TPC compared to control. The difference between 200 kg ha^{-1}, 400 kg ha^{-1}, and 600 kg ha^{-1} humic acid was not significant. Tohidi et al. (2017) reported different levels of total phenolics in a trial with nine different *Thymus* species from different regions of Iran. Accordingly, total phenolic contents of *T. migricus*, *T. fallax*, *T. serpyllum*, *T. trautvetteri*, *T. transcaspicus*, *T. carmanicus*, *T. fedtschenkoi*, *T. daenensis*, and *T. pubescence recorded* were reported as 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 and 62.40 (mg TAE g DW^{-1}), respectively. Özyazıcı et al. (2018) investigated the effects of five harvest day-times on some biochemical properties of garden thyme. According to their result, the highest content of polyphenols for garden thyme was found at 6:00 (42.66 mg gallic acid g DW^{-1}). Nickavar and Esbati (2016), reported that TPC was 295.93, 337.00, and 295.57 (µg rutin/mg extract) on three *Thymus* species, *T. daenensis*, *T. kotchyanus*, and *T. pubescens* respectively. Amzad Hossain et al. (2013) indicated that among the five crude extracts, butanol extract contained the highest (245.26 mg g^{-1}) amount of phenol compounds followed by hexane extract (160.35 mg g^{-1}), chloroform extract (158.5 mg g^{-1}), ethyl acetate extract (84.85 mg g^{-1}), and methanol (49.43 mg g^{-1}). Eghdami et al. (2013), reported that phenolic contents in *T. vulgaris* were 23.34±1.3 mg GAE g DW^{-1} in 50% methanolic extract. Georgieva and Mihaylova, (2015), reported that phenolic contents in *T. vulgaris* were 25.20±0.57 mg GAE g DW^{-1} in methanolic extract. Medicinal and aromatic plants are one of the best sources of chemical ingredients, antimicrobial and antioxidant agents for the cure of different diseases. Without paying attention to the effect of humic acid crude extracts from the plant showed good amounts of total phenol.

3.2. Total flavonoids content

The results showed that TFC in the leaves of the plant was significantly affected by different levels of humic acid in the first and second harvest ($p< 0.01$). At the first harvest, the highest TFC was recorded in the 600 kg ha^{-1} treatment (0.794±0.06 mg quercetin g DW^{-1}) and the lowest was in the

Table 3. Mean comparison of antioxidant activity from in the first harvest of *T. vulgaris* L. affected by humic acid*

Humic acid doses (kg ha^{-1})	Total phenols content (mg gallic acid g DW^{-1})	Total flavonoids content (mg quercetin g DW^{-1})	Nitric oxide radical scavenging activity (%)	DPPH radical scavenging activity (%)	Chain breaking activity (-Abs-3 /min/mg extract)
Control	28.71±1.22	0.481±0.06 b	30.66±4.07 a	91.33±1.91	58.56±6.62
200	28.87±1.34	0.498±0.05 b	14.45±3.63 b	89.89±3.29	60.88±3.21
400	28.85±2.04	0.766±0.09 a	15.48±2.00 b	91.71±3.63	59.76±4.54
600	29.34±1.61	0.794±0.06 a	14.76±5.13 b	92.20±1.81	59.65±3.61
CV (%)	5.92	12.44	19.79	2.14	4.06
P	0.957	0.0004	0.0004	0.425	0.627

*: The difference between the means indicated by the same letter in the same column is not significant, CV: Coefficient of variation, P: Significant level

Table 4. Mean comparison of antioxidant activity from in the second harvest of *T. vulgaris* L. affected by humic acid*

Humic acid doses (kg ha^{-1})	Total phenols content (mg gallic acid g DW^{-1})	Total flavonoids content (mg quercetin g DW^{-1})	Nitric oxide radical scavenging activity (%)	DPPH radical scavenging activity (%)	Chain breaking activity (-Abs-3 /min/mg extract)
Control	35.38±1.81 a	0.511±0.08 c	19.64±5.22	57.84±9.23 b	28.53±2.69 c
200	26.66±2.03 b	0.702±0.06 b	19.46±3.69	56.71±6.81 b	31.76±5.08 bc
400	26.21±1.50 b	0.758±0.06 ab	19.00±4.47	54.38±6.24 b	41.64±6.56 a
600	27.04±1.49 b	0.813±0.02 a	18.84±5.76	75.50±10.70 a	40.03±9.39 ab
CV(%)	4.28	7.90	27.43	14.50	16.64
P	0.0001	0.0002	0.995	0.0291	0.0321

*: The difference between the means indicated by the same letter in the same column is not significant, CV: Coefficient of variation, P: Significant level

control treatment (0.481±0.06 mg quercetin g DW^{-1}) (Table 3). The difference between 400 kg ha^{-1} and 600 kg ha^{-1} humic acid was not significant. Also, the difference between control and 200 kg ha^{-1} humic acid was not significant. In the second harvest, the highest TFC was recorded in the 600 kg ha^{-1} treatment (0.813±0.02 mg quercetin g DW^{-1}) and the lowest was in the control treatment (0.511±0.08 mg quercetin g DW^{-1}) (Table 4). The difference between 400 kg ha^{-1} and 600 kg ha^{-1} humic acid was not significant. Tohidi et al. (2017) indicated that flavonoid contents 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^{-1}) respectively. Ghandchi and Jamzad (2015), reported that total flavonoid contents of *T.* trautvetteri in different solvents were (2.076%, 1.468%, and 1.412%) mg g^{-1}. Eghdami et al. (2013), reported that flavonoids content in *T. vulgaris* was 4.303±0.05 QE g^{-1} in methanolic extract. Kruma et al. (2008), reported that the TFC extracted with methanol in garden thyme was 0.376 mg g^{-1}. 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-allergic (Amzad Hossain et al., 2013). Amzad Hossain et al. (2013) reported that among the five crude extracts of *T. vulgaris*, methanol extract contained the highest (1,71 mg g^{-1}) amount of flavonoids content compounds followed by butanol (1.55 mg g^{-1}), chloroform (1.37 mg g^{-1}), ethyl acetate (1.29 mg g^{-1}) and hexane (1.18 mg g^{-1}). Rahimi et al. (2018a), evaluated the effects of four drying methods on garden thyme on some biochemical properties. According to their results, flavonoid content ranged from 0.53 to 0.63 mg quercetin g DW^{-1} in different drying methods.

3.3. Nitric oxide radical scavenging activity

According to the results, nitric oxide radical scavenging activity in the leaves of garden thyme was significantly affected by different levels of humic acid in the first harvest ($p<0.01$) whereas in the second harvest it was not significant (Table 3 and 4). The highest nitric oxide radical scavenging activity was recorded in control (30.66±4.07%) and the lowest was in 200 kg ha^{-1} humic acid (14.45±3.63%) (Table 3). The difference between 200 kg ha^{-1} and other humic acid levels was not significant. According to the results using different levels of humic acid reduced nitric oxide radical scavenging activity compared to control. Nitric oxide radical scavenging activity ranged from 18.84±5.76 to 19.64±5.22% at the second harvest (Table 4). Tchamgoue et al. (2015), in research about garden thyme, indicated that nitric oxide scavenging activity in the seeds of the plant (extracted by methanol) ranged from 8.12% to 35.67%. Parul et al., (2012) reported that nitric oxide radical scavenging activity in leaves of *Triumfetta rhomboidae* by the methanolic extracts was 20.294 to 53.942% in different concentrations. Rahimi et al. (2018a) stated that there was no statistically significant difference in garden thyme plants according to the drying methods, and the radical scavenging activity ranged between 18.41% and 20.75%. On the other hand, Özyazıcı et al. (2018), reported that the nitric oxide radical scavenging activity in *T. vulgaris* varied from 14.48% to 24.72% according to different harvest day-times, and there was a statistically significant difference between harvest day-times.

3.4. DPPH radical scavenging activity

The results indicated that DPPH radical scavenging activity (%) of leaves of garden thyme was significantly affected by different levels of humic acid in the second harvest ($p<0.05$) whereas in the first harvest it was not significant. In the first harvest, DPPH radical scavenging activity in different levels of humic acid ranged from 89.89±3.29% to 92.20±1.81% (Table 3). The highest DPPH radical scavenging activity was recorded in 600 kg ha^{-1} humic acid (75.50±10.70%) in the second harvest (Table 4). The difference between control, 200 kg ha^{-1} humic acid and 400 kg ha^{-1} humic acid treatments were not significant. Eghdami et al. (2013), reported that DPPH radical scavenging activity in *Thymus vulgaris* was 66.82±6.0 in 50% methanolic extract and 25.2±0.75% in methanolic extract. Georgieva and Mihaylova (2015), reported that DPPH radical scavenging activity in *T. vulgaris* was 7.97±0.78 µMTE g DW^{-1} in methanolic extract. Istrati et al. (2013) reported that DPPH radical scavenging activity (%) in *T. vulgaris* was 75.86 ± 0.62 when extracted with water. Aazza et al. (2011) in a research on some aromatic plants investigated their antioxidant activity; according to their results, DPPH in *T. vulgaris* was 0.259 (EC50 mg mL^{-1}). According to the results, all the samples from different micronutrients treatments possessed potent free radical scavenging and antioxidant activities in different assays. Asensio-S.-Manzanera et al. (2011) indicated that DPPH in ten populations of *T. mastichina* collected from different regions of Spain in 2009, ranged from 1.78 to 0.59 (EC50 mg mL^{-1}).

3.5. Chain-breaking activity

The results showed that the CBA of the leaves of garden thyme was significantly affected by different levels of humic acid in the second harvest ($p<0.05$) whereas it was not in the first harvest (Table 3 and 4). In the first harvest, CBA varied from 58.56±6.62 to 60.88±3.21 -Abs-3 /min/mg extract) (Table 3). The highest percentage of the character was recorded in the 400 kg ha^{-1} humic acid treatment (41.64±6.56 -Abs-3 /min/mg extract) and the lowest was related to the control treatment (28.53±2.69 -Abs-3 /min/mg extract) in the second harvest. In terms of CBA, there was no significant difference between 400 kg ha^{-1} and 600 kg ha^{-1} humic acid treatments (Table 4). The chain-breaking activity of *T. vulgaris* has been reported to range between 26.81 and 65.41 (Taghipour et al., 2017; Özyazıcı et al., 2018; Rahimi et al., 2018a, 2018b).

4. Conclusions

The effect of humic acid on antioxidant activity in the leaves of garden thyme generally was more effective in the second harvest than the first. The use of humic acid at various concentrations increased total flavonoids content, DPHH radical scavenging activity, and chain-breaking activity. The DPPH method for antioxidant activity shows the ability of the present compounds to scavenge hydrophilic free radicals. Garden thyme contributes to antioxidant activity through flavonoids. It seems that the application of humic acid increased the antioxidant activity by increasing the content of this antioxidant.

Declaration of Author Contributions

Investigation, Data Curation, Formal Analysis, Visualization, and Writing-Review & Editing*, M. MIRZAPOUR*; Conceptualization, Material, Methodology, Visualization, Supervision, Project Administration, Funding Acquisition, and Writing-Review & Editing, *A. RAHIMI*; Project Administration and Writing-Original Draft Preparation, *S. HEYDARZADEH*. All authors declare that they have seen/read and approved the final version of the article ready for publication.

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Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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