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Original Article

The Effect of Micronutrients Supplementation (Fe, Zn, B, and Mn) on Antioxidant Activity of Milk Thistle (*Silybum marianum* L.) under Rainfed Condition

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Abstract

Milk thistle extract is used to treat liver diseases. The aim of this experiment was to study the effects of micronutrients (Fe, Zn, B, and Mn) on antioxidant activity of milk thistle seed. 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 2016-2017, used randomized complete block design in three replications. Control, Fe, Zn, B and Mn were used as fertilizer treatments. According to the results the effect of micronutrients on total phenols and flavonoids content, nitric oxide radical scavenging, super oxide radical scavenging activity, DPPH radical scavenging activity, and Chain-breaking activity was significant ($P \leq 0.01$). Total phenolic content under Zn spraying had significant difference than other treatments whereas in terms of flavonoids content, Zn and B spraying was significant. DPPH radical scavenging activity was higher in Mn and Fe using than other treatments as the difference was significant. Application of mentioned micronutrients significantly decreased the percentage of super oxide and nitric oxide radical scavenging activity than control. In general, according to the findings of this study, spraying micronutrients such as Fe, Zn, B and Mn can be useful to increase the antioxidant capacity of milk thistle under dryland conditions.

Keywords: Antioxidant, Drought stress, Micronutrients, Milk thistle

Introduction

Many antioxidant compounds such as phenols, polyphenols, flavonoids, and terpenes which have been isolated from plants identified to play a protective role in the human and animal system. Antioxidants are also useful against environmental stress. Increasing consumption of dietary antioxidant compounds can help to maintain a sufficient antioxidant status [1,2]. Several common plants are rich sources of antioxidants which have a high content of flavonoids; or some which possess high amounts of quercetin and protocatechuic acid [3]. Wild plants are better sources of antioxidant compounds than cultivated plants, because they grow under hard conditions [4].

Milk thistle (*Silybum marianum* L.) as folk medicine using, is an annual or biennial plant of Asreraceae family; the plant is native to the Mediterranean, but now widespread all over the world [5]. The plant's common name comes from the white markings on the leaves and its milky white sap used traditionally by nursing mothers to increase milk [6]. The seeds of the plant contain 20-35% fatty acids, flavonoids, and other polyphenolics and 65-85% flavonolignans like isosilychristin, silydianin, silychristin, silybin A and B, and isosilybin A and B. The main source of silymarin is seed [7]. The component has been widely studied for hepatoprotective properties and several putative hepatoprotective mechanisms [8]. The component directly helps hepatocytes by binding to the outside of the cells and blocking the binding of potential hepatocellular toxins [9]. The plant extract was used from ancient time for liver diseases such as hepatitis, icterus, and cyrosis, against the intoxication, and in the peoples with alcoholic problems too [7]. Silymarin is an effective free radical scavenger and has been well-known to increase production of glutathione in hepatocytes [10]. In

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addition this effective property of the extract is due to the antioxidant properties of flavonoids, to the inhibition of the synthesis of phosphatidylcholine, and stimulating of hepatic synthesis of RNA proteins [7].

Micronutrients used in lower amount compared to macronutrients. These nutrients are crucial substances for plant's growth. They possess a chief role in development of meristematic tissues, cell division, respiration, photosynthesis, and acceleration of plant maturity. Micronutrients such as Fe, B, Zn, and Mn are considered vital for plants animals, and humans. These nutrients can maintain crop-physiology balance due to these nutrients play vital roles in vitamin A improvement, CO_2 flowing out, and resistant system doings [11,12].

Iron (Fe) is vital for photosynthesis and chlorophyll formation; the element is important in the enzyme systems and respiration in higher plants. Zinc (Zn) is critical for enzymes production and sugar regulation which control plant growth. Manganese (Mn) is involved in the enzyme systems related to carbohydrate [13]. Boron (B) made resistance of plasmalemma in cells. This micronutrient increase resistance combination by other minerals and necessary for plants [14].

The reports about the effect of micronutrients on antioxidant activity of milk thistle seed are scarce. It is believed that this study will be a good source for future researches. The main objective of the submitted work was to evaluate the effect of some micronutrients (Fe, Zn, B and Mn) on antioxidant activity of milk thistle in Urmia condition, West Azerbaijan, Iran. The extracts of milk thistle seed were evaluated to determine the total amount of phenol, flavonoid, chain-breaking activity (CBA), DPPH (1,1-diphenyl 2-picryl hydrazyl) radical scavenging activity, nitric oxide radical scavenging activity, and superoxide radical scavenging activity.

Material and Methods

This study was conducted at the experimental fields of the Department of Agronomy, Faculty of Agriculture (latitude 37.39° N, 44.58° E, and 1365 meter above sea level) and the Lab of Biology Department, Urmia University, Urmia, Iran, during 2016-2017. The trial arranged in a randomized complete block design with three replications in plots of an area of 6 m^2. The long term outdoors climatic data of the experimental city (Fig 1) are shown. 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 60 cm. The seeds for sowing were obtained from Ankara University, Ankara, Turkey. Sowing was carried out at the field in 16. 03. 2016. Nitrogen fertilizer was used in sowing time, and vegetative phase according to soil analysis.

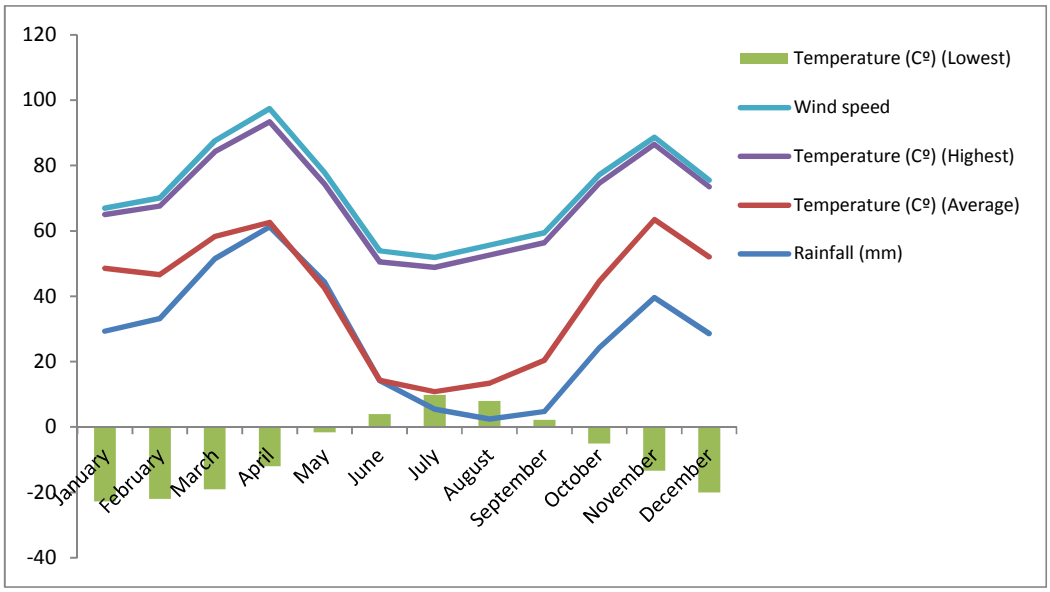

Fig. 1 The long term outdoors climatic data of the experimental city

Table 1 Soil analyses results of the experimental soil samples in the field before corm sowing

EC	$CaCO_3$	B.S	F.C	Clay	Loam	Sand	Texture
1.32 dSm^{-1}	16.3%	49%	27%	44%	34%	22%	Clay-Loam
O.C	pH	K	P	Fe	Zn	B	Mn
1.18%	7.7	320 mg kg-1	9.54 mg kg-1	17 mg kg-1	1.6 mg kg-1	0.4 mg kg-1	15 mg kg-1

Foliar application of micronutrients including: control, Fe, Zn, B and Mn. Foliar application of micronutrients was done at two times: 1) early of vegetative phase, and 2) early of generative phase or flowering stage. Harvestings were done seed raping.

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 1) are shown.

Preparation of Extracts

The seeds of milk thistle were powdered with a grinder. Extraction procedure involved the addition of 25 mL methanol as 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 and stored at 4 °C until experiments in the dark [15].

Measurements

Total phenolic Content Determination (TPC)

Total phenolic contents of extracts were estimated by the Folin-Ciocalteu colorimetric method described previously [16] with a little modification. Folin Ciocalteu's phenol reagent (1 mL) and 10% w/v Na_2CO_3 (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 the seed powder **(**The calibration equation for Gallic acid: y= 0.0415x- 0.0163).

Total Flavonoid Content (TFC)

Total flavonoid contents of extracts were determinded by aluminum chloride colorimetric method described previously [17] with a little modification. Brifley,10 µl of 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 % $AlCl_3$ 6H2O 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 the seed powder **(**The calibration equation for Gallic acid: y= 0.0772x- 0.0084).

DPPH Radical Scavenging Activity

The free radical scavenging activity of plant extracts were determined by slight medications of the method described previously [18]. 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

In this equation, A blank is the adsorption of the reaction mixture without the extract and A sample is the adsorption of the reaction mixture containing the extract.

Nitric Oxide Radical Scavenging Activity (NORSA)

Nitric oxide radical inhibition can be estimated by the use of Griess Ilosvay reaction [19]. 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 milk thistle 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 blank

In this equation, A blank is the adsorption of the reaction mixture without the extract and A sample is the adsorption of the reaction mixture containing the extract.

Chain-breaking Activity (CBA)

The Chain-breaking activity was based on the method of Brand-Williams *et al*. [20] 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

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as k. Antioxidant activity was reported as (-Abs^{-3} /min/mg extract).

Super Oxide Radical Scavenging Activity (SORSA)

For superoxide anion radical assay, the superoxide anion radicals were generated by a pyrogallol autoxidation system [21]. A volume of 9 mL of Tris–HCl buffer solution (50 mmol/L, pH 8.2) was added into a test tube, and the test tube was incubated in a water bath at 25 C for 20 min. A volume of 40 lL of pyrogallol solution (45 mmol/L of pyrogallol in 10 mmol/L of HCl), which was also pre-incubated at 25 C, was injected to the above test tube with a microlitre syringe and mixed up. The absorbance at 420 nm was measured 5 min later, and this denotes the speed of pyrogallol autoxidation. Ascorbic acid was used as positive control. The autoxidation speed was obtained by applying the above method and with the addition of a certain concentration of extract and positive control into the Tris–HCl buffer solution. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The superoxide radical scavenging activity was calculated according to the following formula:

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

In this equation, A blank is the adsorption of the reaction mixture without the extract and A sample is the adsorption of the reaction mixture containing the extract.

Statistical Analysis

The analysis of variance (ANOVA, one-way analysis) was performed using SAS 9.1 to detect the significance of differences among the treatment means. Means comparison of traits was performed using Duncan new multiple range.

Results and Discussion

The results showed that the effect of micronutrients on total phenols content, and flavonoids content, nitric oxide DPPH, super oxide radical scavenging activity, and chain-breaking activity was significant ($P \leq 0.01$) (Table 2). The highest total phenols content in the seeds of milk thistle recorded in the Zn (32.34 mg Gallic acid/g DW) and the lowest was related to the B (29.15 mg Gallic acid/g DW).

Table 2 Effect of micronutrients on antioxidant properties of milk thistle

Source of variation	df	Mean Squares					
		TPC	TFC	NORSA	DPPH	CBSA	SORSA
Block	2	0.015	0.001	0.062	0.075	0.019	0.769
micronutrients	4	4.536 **	0.220 **	4.075 **	7.208 **	0.134 **	52.335 **
Error	8	0.011	0.003	0.230	0.171	0.012	1.372
Coefficient variation (%)		0.339	2.366	1.613	0.740	8.062	0.772

** : Significant at levels of probability 5% .

Total Phenolic Content in the Seeds of Milk Thistle

According to the results using micronutrients such as Zn increased total phenolic content than control whereas using Mn and B reduced (Fig. 2). Total phenolic content in Zn treatment had significant difference than other treatments. Phenols have antioxidant property, so they scavenge and reduce reactive oxygen species (ROS), thereby preventing the oxidation of vital biomolecules of cells and avoid oxidative stress and/or mitigate its impacts on plant cells. The phenolic content of milk thistle was higher than other species of the Asteraceae family cited in the literature, the leaves of *Achillea millefolium* (9.55 mg GAE/g DW), the aerial part of *Artemisia vulgaris* (3.83 mg GAE/g DW) and leaves of *Tanacetum vulgare* (1.68 mg GAE/g DW) [26]. Sun *et al.* [27] indicated that the pappi exhibited the highest the total phenolic content (48.97 mg GAE/g DW), followed by the fruit receptacles (22.19 mg GAE/g DW); the lowest was in the main stem (9.8 mg GAE/g DW). Salem *et al*. [28], investigated on the total phenolic content in different parts of milk thistle (methanolic extracts); they indicated that total phenolic content in leaves, flowers, and stem were 32.17, 22.19, and 35.60 (mg GAE/g DW) respectively. Tupe *et al.* [29] in a trial on different medicinal plants reported that the total phenolic content of milk thistle seeds (methanolic extracts) was 18.33 (µg GAE/mg DW). Serçe *et al*. [30] indicated that total phenolic content of ethanol extract of milk thistle seeds was 62.0 (mg GAE/g DW). The phenolic contents (expressed as GAE was found to be 23.26 (mg GAE/g DW) in infusions, and 20.92 in dietary supplements, respectively [31]. Yadegari [32] researched about the effect of micronutrient on total phenols content of *Borago officinalis* and indicated that foliar application of Mn, Fe and Zn increased total phenols content than control in the plant. 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 [33]. fertilizers in combination or alone can increase the amount of biochemical compounds of plants such as phenol and flavonoids. Combined biofertilizers with compost increase the biochemical properties of the plants compared to organic and Inorganic Fertilizers [44].

Flavonoids Content in the Seeds of Milk Thistle

The highest total flavonoids content was recorded in the B foliar application (2.79 mg quercetin/ g DW) and the lowest was related to the Fe and Mn treatments (Fig. 2). Total flavonoids content in B foliar application had significant difference than other treatments. Serçe *et al.* [30] investigated on milk thistle seeds and reported that the flavonoid content (expressed as QE) was 39.32 µg/g. Pereira *et al.* [31] described the content of flavonoids in milk thistle. In their study, milk thistle plant was used as dry material for infusion and pill preparation.

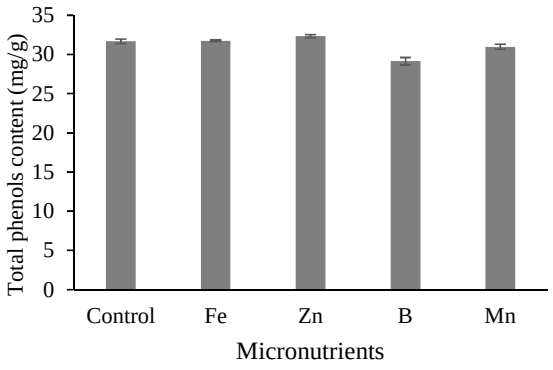

Fig. 1 The effect of micronutrients on total phenols content of milk thistle seeds. Different letters mean significant differences according to Duncan's multiple range test at p≤0.01.

In milk thistle flavonoid contents (expressed as catechin equivalent) was 6.95 mg/g in infusions, and 3.88 mg/g in dietary supplements, respectively [31]. Sun *et al.* [34], investigated on the total flavonoid content (TFC) in the roots, main stems, leaves, fruit receptacles, and pappus of milk thistle; They indicated that the pappus exhibited the highest TFC (17.10 mg rutin/g DW), followed by the fruit receptacles (15.34 mg rutin/g). Salem *et al.* [28] indicated that the total flavonoid content (TFC) (methanolic extracts) in leaves, flowers, and stem were 3.90, 3.82, and 8.31 mg quercetin/ g DW respectively. Yadegari [32], showed that foliar application of B and Mn was significantly increased total flavonoids content in plants of *Calendula officinalis* L. Flavonoids are very important plant phytochemical components due to their radical scavenging properties and there are widely in plants. Flavonoids in medicinal plants as one of the most diverse and widespread group of natural compounds, specially possess a broad spectrum of chemical and biological activities. It is well known that flavonoids have anticarcinogenic activity, antiallergenic, antiviral, antiageing and anti-inflammatory properties [22,25,35].

Nitric Oxide Radical Scavenging Activity (%) in the Seeds of Milk Thistle (NORSA)

The analysis of variance revealed that nitric oxide radical scavenging activity was significantly ($P \leq 0.01$) affected by the micronutrients. The comparison of the means showed that the highest nitric oxide radical scavenging activity was obtained in control (31.14%). The lowest amount was related to Mn foliar application by 28.13% (Fig. 3). In terms of the character, control had significant difference than Fe, Mn and Zn foliar application. As the results using micronutrients (Fe, Zn, and Mn) significantly reduced nitric oxide radical scavenging activity in the plant seeds. Low concentration nitrogen oxide can protect the cell against oxidation, but at high levels, in combination with H_2O_2, it can have detrimental effects on the cells, which causes toxicity. 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 [37,38]. This toxicity leads to membrane damage (MDA), and DNA disorder. Natural plant extracts and antioxidants scavenge these toxic effects. Tupe *et al.* [29], in an experiment on different medicinal plants reported that the nitric oxide radical scavenging activity of milk thistle seeds was 34.69%. The interactions of NO with reactive oxygen species (ROS) such as H_2O_2 and O_2^- can be protected or cytotoxic [36].

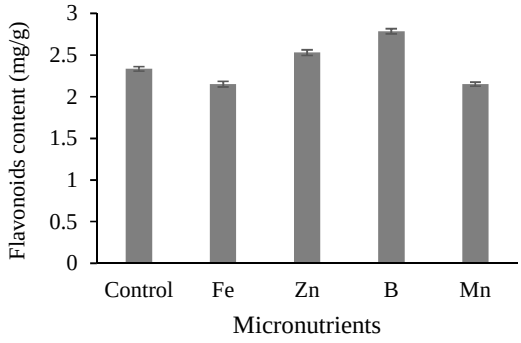

Fig. 2 The effect of micronutrients on flavonoids content of milk thistle seeds. Different letters mean significant differences according to Duncan's multiple range test at p≤0.01.

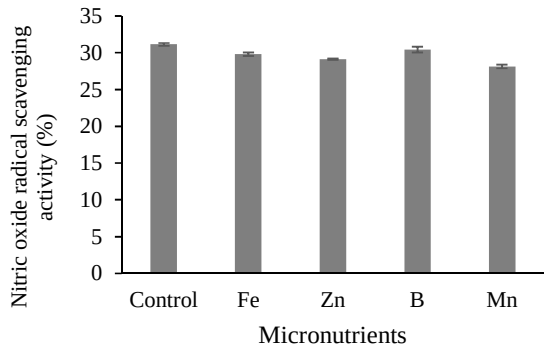

Fig. 3 The effect of micronutrients on nitric oxide radical scavenging activity of milk thistle seeds. Different letters mean significant differences according to Duncan's multiple range test at p≤0.01.

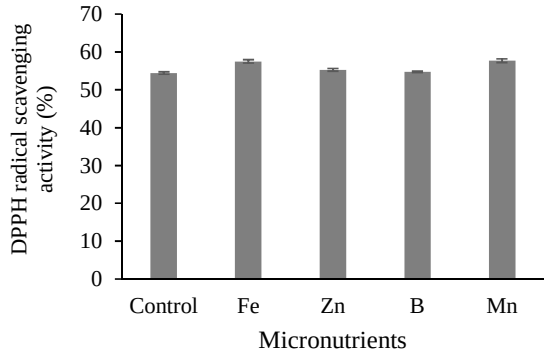

Fig. 4 The effect of micronutrients on DPPH radical scavenging activity of milk thistle seeds. Different letters mean significant differences according to Duncan's multiple range test at p≤0.01.

DPPH Radical Scavenging activity (%) in the Seeds of Milk Thistle

The highest DPPH radical scavenging activity was obtained in Mn foliar application (57.70%) and the lowest percentage related to control (54.47%) as same as Zn and B foliar application (Fig. 4). As the results Fe and Mn significantly increased the character than control. Sun *et al*. [27], investigated on DPPH radical scavenging activity in the roots, main stems, leaves, fruit receptacles, and pappus of milk thistle; At 50 ug/mL, the pappus ethanol extract showed the highest DPPH radical scavenging activity (69.68%), followed by the roots (66.02%). Tupe *et al*. [29], reported that the DPPH radical scavenging activity of milk thistle seeds was 92.45%. Farnad *et al*. [15] reported that the highest antioxidant properties of peppermint (*Mentha piperita*) in methanol extract was 66.98 %. The stable free radical DPPH method is an easy, rapid, and sensitive way to survey the antioxidant activity of specific compounds or plant extracts [39]. Numerous studies have shown that organic and biological fertilizers alone or in combination with chemical fertilizers can increase the amount of secondary metabolites and DPPH radical scavenging activity [40-42].

Chain-breaking Scavenging Activity in the Seeds of Milk Thistle (CBSA)

Variance analyses of the results indicated that Chain-breaking scavenging activity (%) of the seeds of milk thistle was significantly affected by micronutrients ($P \leq 0.01$) (Table 2). According to the results, chain-breaking scavenging activity in different treatments ranged from 11.87 to 17.20 (-Abs-3 /min/mg extract) (Fig. 5). The highest amount of the character was recorded in Fe treatment (17.20 -Abs-3 /min/mg extract) and the lowest related to in control (11.87 -Abs-3 /min/mg extract) (Fig. 5). Chain-breaking scavenging activity increased only in

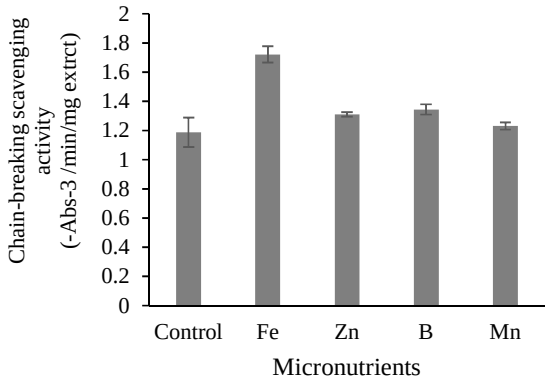

Fig. 5 The effect of micronutrients on Chain-breaking scavenging activity of milk thistle seeds. Different letters mean significant differences according to Duncan's multiple range test at p≤0.01.

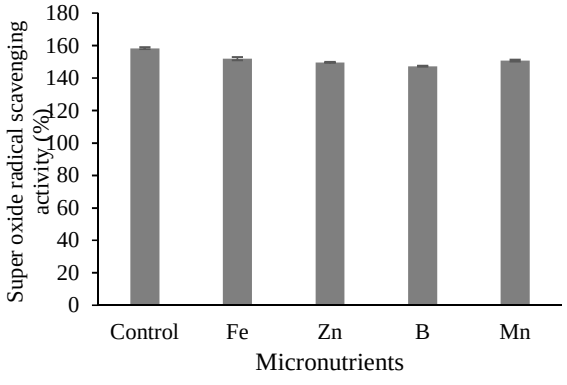

Fig. 6 The effect of micronutrients on super oxide radical scavenging activity of milk thistle seeds. Different letters mean significant differences according to Duncan's multiple range test at p≤0.01.

Fe foliar application than control. Other micronutrients foliar application as Zn, B and Mn showed non-

significant difference with control. The measurement of the chain breaking activity determines the rate of radical scavenging as influenced by electron-transferring and hydrogen-donating antioxidants. The chain breaking have correlation with amount of antioxidants which are boosted by different fertilizers. Therefore, the measurement of these parameters together can be an interesting way to estimate the antioxidant capacity of a compound [43].

Conclusions

Super Oxide Radical Scavenging Activity (%) in the Seeds of Milk Thistle (SORSA)

According to the analysis of variance, the spray of micronutrient treatments was significant ($P \leq 0.01$) for superoxide radical scavenging activity (Table 2). Super oxide radical scavenging activity in different treatments ranged from 147.32% to 158.44% (Fig. 6). The highest percentage of the character was recorded in control (158.44%) and the lowest related to in B foliar application (147.32%) (Fig. 6). In terms of the character, the control had significant difference than other treatments. Using micronutrients such as Fe, Zn, B and Mn exhibited more difference of super oxide radical scavenging activity with control plants. The superoxide anion radical is produced via mitochondrial respiration. High concentration of superoxide anions causes formation of other reactive oxygen species. These anions effect on the physiological function of cell. Plant antioxidant extracts have ability to scavenge the superoxide radicals and as mentioned in various sources, the amount of these antioxidants can be increased by different fertilizers [15].

According to the results, micronutrient treatments had different effects on antioxidant properties of the milk thistle seeds. Application of Fe and Zn lifted total phenolic content than control, whereas Mn and B reduced. Using of Zn and B increased flavonoid content but Fe and Mn decreased. In terms of nitric oxide radical scavenging activity, control was the best; in other word micronutrients had negative effect on the character. Mn and Fe improve the percentage of DPPH radical scavenging activity in the seeds than control. Chain-breaking activity increased by using Fe than control whereas other micronutrients had not effective role. In terms of super oxide radical scavenging activity, control is the best than other micronutrients application.

Referencess

1. Schandalik R, Gatti G, Perucca E. Pharmacokinetics of silybin in bile following administration of silipide and silymarin in cholecystectomy patients. Arzneimittelforschung. 1992;42:964-968.
2. Kelly FJ. Dietary antioxidants and environmental stress. Proc Nutr Soc. 2004;63:579-585.
3. Ly TN, Hazama C, Shimoyamada M, Ando H, Kato K, Yamauchi R. Antioxidative compounds from the outer scales of onion. J Agric Food Chem. 2005;52:8183-8189.
4. Salvatore S, Pellegrini N, Brenna OV, del Río D, Frasca G, Brighenti F, Tumino R. Antioxidant characterization of some Sicilian edible wild greens. J Agric Food Chem. 2005;53:9465-9471.
5. Young JA, Evans RA, Hawkes RB. Milk Thistle (Silybum marianum L.) Germination. Weed Sci. 1978;26:395-398.
6. Presser A. Pharmacist's Guide to Medicinal Herbs. Smart Publications, Petaluma, CA. 2000;259-260.
7. Ramasamy K, Agarwal R. Multitargeted therapy of cancer by silymarin. Cancer Lett. 2008;269:352-362.
8. Tsai JH, Liu JY, Wu TT, Ho PC, Huang CY, Shyu JC. Effects of silymarin on the resolution of liver fibrosis induced by carbon tetrachloride in rats. J Viral Hepatitis. 2008;15:508-514.
9. Tuchweber B, Sieck R, Trost W. Prevention by silibinin of phalloidin induced hepatotoxicity. Toxicol Appl Pharmacol. 1979;51:265-275.
10. Magliulo E, Carosi PG, Minoli L, Gorini S. Studies on the regenerative capacity of the liver in rats subjected to partial hepatectomy and treated with silymarin. Arzneimittelforschung. 1973;23:161-167.
11. Mohamed M, El-Fouly Zeinab M, Mobarak and Zeinab A, Salama M. Micronutrients (Fe, Mn, Zn) foliar spray for increasing salinity tolerance in wheat Triticum aestivum L. Afr J of Plant Sci. 2011;5:314-322.
12. Welch RM, Allaway WH, House WA, Kubota J. Geographic distribution of trace element problems. In: Mortvedt JJ, ed. Micronutrients in agriculture, 2nd Ed. Madison, Wisconsin: 1991; SSSA Book Ser. 4. SSSA:31-57.
13. Havlin JL, Beaton JD, Tisdale SL, Nelson WL. Soil Fertility and Fertilizers – An introduction to nutrient management 6th Ed. Prentice Hall, New Jersey. 1999;1034-1056.
14. Widom M, Mihalkovic M. Symmetry-broken crystal structure of elemental boron at low temperature. Phys Rev B. 2008;77:24-34.
15. Farnad N, Heidari R, Aslanipour B. Phenolic composition and comparison of antioxidant activity of alcoholic extracts of Peppermint (Mentha piperita). J. Food Meas Charact. 2014;8:113-121.
16. Kahkonen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS. Agric J. Food Chem. 1999;57:792-797.
17. Youngjae LRS, Hai F, Jacqueline DH, Nockc BW. Christopher, Postharvest. Biol Technol. 2007;45:349-357.
18. Hatano T, Kagaw H, Yasuhar T, Okuda Chem T. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. Pharm Bull. 1988;36:1090-2097.
19. Garrat DC. The quantitative analysis of drugs (Chapman and Hall Ltd, Japan,). 1964;211-214.
20. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensm Wiss Technol. 1995;28:5-30.
21. Jing TY, Zhao XY. The improved pyrogallol method by using terminating agent for superoxide dismutase measurement. Prog Biochem Biophys. 1995;22:84-86.

22. Altaş S, Kızıl G, Kızıl M, Ketani A, Haris PH. Protective effect of Diyarbakır watermelon juice on carbon tetrachloride–induced toxicity in rats. Food Chem Toxicol. 2011;49:2433-2438.
23. Dapkevicius A, Van Beek TA, Lelyveld GP, Van Veldhuizen A, De Groot AE, Linssen JPH, Venskutonis R. Isolation and structure elucidation of radical scavengers from Thymus vulgaris leaves. J Nat Prod. 2002;65:892-896.
24. Shahidi F, Wanasundara PKJPD. Phenolic antioxidants. Crit Rev Food Sci Nut. 1992;32:67-103.
25. Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol. 1982;32:1141-1148.
26. Wojdyło A, Oszmian´ski J, Czemerys R. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem. 2007;105:940-949.
27. Sun J, Li X, Yu X. Antioxidant Activities, Total Flavonoids and Phenolics Content in Different Parts of Silybum Marianum L. Plants. Chem Eng Trans. 2016;55:37-42.
28. Salem N, Dhifi W, Graya A, Mnafeg F, Gharbi S, Khammassi S, Marzouk B, Limam F, Chekir Ben Chaouacha R. Antioxydant activity of Silybum marianum and Ajuga iva natural dyes. Int J Control Autom Syst. 2016;3:6-12.
29. Tupe RS, Kemse NG, Khaire AA. Evaluation of antioxidant potentials and total phenolic contents of selected Indian herbs powder extracts. Int Food Res J. 2013;20:1053-1063.
30. Serçe A, Çeken Toptancı B, Emen Tanrıkut S, Altaş S, Kızıl G, Kızıl S, M. Assessment of the Antioxidant Activity of *Silybum marianum* Seed Extract and Its Protective Effect against DNA Oxidation, Protein Damage and Lipid Peroxidation, Food Technol Biotechnol. 2016;54:455-461.
31. Pereira C, Calhelha RC, Barros L, Queiroz MJRP, Ferreira ICFR. Synergism in antioxidant and anti–hepatocellular carcinoma activities of artichoke, milk thistle and borututu syrups. Ind Crops Prod. 2014;52:709-13.
32. Yadegari M. Foliar application of micronutrients on essential oils of borago, thyme and marigold. J Soil Sci Plant Nutr. 2015;15:949-964.
33. Vaya J, Belinky PA, Aviram M. Antioxidant constituents from licorice roots: Isolation, structure elucidation and antioxidative capacity toward LDL oxidation. Free Radic Biol Med. 1997;23:302-313.
34. Sun J, Li X, Yu X. Polysaccharides, total flavonoids content and antioxidant activities in different parts of Silybum marianum L. plants, International Conference on Materials Science, Resource and Environmental Engineering, AIP Conf Proc. 2016;1794:050004-1-050004-4.
35. Jamshidi M, Ahmadi HR, Rezazadeh S, Fathi F, Mazanderani M. Study on phenolic and antioxidant activity of some selected plant of Mazandaran province. Medic Plan. 2010;9:177-183.
36. Beligni MV, Fath A, Bethke PC, Lamattina L, Jones RL. Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. Plant Physiol. 2002;129:1642-1650.
37. Wink DA, Miranda KM, Espey MG, Pluta RM, Hewett SJ, Colton C, Vitek M, Feelisch M, Grisham MB. Mechanisms of the antioxidant effects of nitric oxide. Antioxid. Redox Signal. 2001;3:203-213.
38. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev. 1991; 43:109-142.
39. Ebrahimzadeh MA, Pourmmorad F, Hafezi S, 2008. Antioxidant activities of Iranian corn silk. Turk J Biol. 2008;32:43-49.
40. Saharan BS, Nehra V. Plant growth promoting rhizobacteria: a critical review. Life. Sci. Med. Res. 2011;21:30.
41. Saikia LR, Upadhyaya S. Antioxidant activity, phenol and flavonoid content of A. racemosus Willd. a medicinal plant grown using different organic manures. Res. J. Pharmaceutical. Biolog. Chem. Sci. 2011;2:457-463.
42. Ibrahim MH, Jaafar HZ. Photosynthetic capacity, photochemical efficiency and chlorophyll content of three varieties of Labisia pumila Benth. exposed to open field and greenhouse growing conditions. Acta. Physio. Plantarum. 2011;33:2179-2185.
43. Nejatzadeh-Barandozi F, Gholami-Borujeni F. Application of different fertilizers on morphological traits of dill (Anethum graveolens L.). Org. Medic. Chem. Letters. 2014;4:4.
44. Javanmardi J, Khalighi A, Kashi A, Bais HP, Vivanco JM. Chemical characterization of basil (Ocimum basilicum L.) found in local accessions and used in traditional medicines in Iran. J. Agric. Food. Chem. 2002;50:5878-5883.