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Influence of different levels of nitrogen fertilizer on some phytochemical characteristics of artichoke (*Cynara scolymus* L.) leaves

Marziyeh Allahdadi and Parisa Farzane

Abstract
Nitrogen has a key role in proper growth and development of all crops including artichoke. In order to study the effect of different levels of nitrogen fertilizer on morphological and phytochemical characteristics of artichoke plant, an experiment was conducted in a randomized complete block design with three replications in Isfahan, Iran during 2014. Treatments were four levels of nitrogen fertilizer (0, 100, 150 and 200 kg N ha^{-1}) from urea source. The result showed that nitrogen application in higher levels improved the growth of artichoke but reduced total phenol, total flavonoid and antioxidant activity. The highest of leaf length (102 cm), number of leaves per plant (30), plant fresh weight (3494.33 g) and dry weight (804.67 g) was observed in 200 kg N ha^{-1}. Phytochemical characteristics of artichoke decreased by use of chemical fertilizer and The maximum amount of total phenol (99.36 mg GAE g^{-1} DW), total flavonoid (1.30 mg QE g^{-1} DW), DPPH free radical scavenging activity (90.96%) and reducing power (2.29) was observed in control treatment. Also, we have found a positive correlation between the total phenol, total flavonoid content and antioxidant activity in artichoke. Based on the results, in order to avoid negative effects of nitrogen on the quality of artichoke leaves, it is recommended to use 100 kg N ha^{-1} when cultivating artichoke for its medicinal use.

Keywords: Artichoke, antioxidant activity, nitrogen fertilization, total phenol

Introduction
Artichoke (*Cynara scolymus* L.) is a perennial plant from Asteraceae family that used for various purposes such as human food (Gouveia and Castilho, 2012) [20], animal feeding (Sallam *et al.* 2008) [49] and medicinal plant in pharmaceutical industry. Several studies described numerous pharmacological activities associated to artichoke, such as hepatoprotective, antioxidative, anticarcinogenic, hypocholesterolemic, anti-HIV, bile-expelling and urinative effects (Pandino *et al.* 2011; Rondanelli *et al.* 2011; Aksu and Altinterim, 2013) [43, 47, 3]. Furthermore, the different parts of artichoke have the antimicrobial effect on some gram positive and gram negative bacteria (Emanuel *et al*., 2011; Alghazeer *et al*., 2012; Gaafar and Salama, 2013) [18, 4, 19].
The aspect of a quality in medicinal plants is very important (Nithiya *et al*., 2015) [39]. Quality of this products is determined by the presence of secondary metabolites such as saponins, alkaloids, tannins, steroids and phenolic compounds in plants. The qualitative and quantitative of secondary metabolites in various plant parts can be influenced by managing factors or farming practices (Sereme *et al*., 2016) [51]. The increases in secondary metabolites and antioxidant activity may be caused by the presence of various major (i.e. nitrogen, potassium and phosphorus) and minor elements in organic fertilizers and inorganic fertilizers (Ibrahim *et al*., 2013) [24]. High nutrient availability leads to an increase in plant growth and development, but it reduces the allocation of resources to produce secondary metabolites (Tarozzi *et al*., 2006) [54]. Macronutrients have a significant effect on plant growth and polyphenols and antioxidants accumulation (Parr *et al*., 2000) [44]. Among the major nutrients nitrogen is the most important element in plant nutrition and is a primary plant nutrient to achieve maximum yield in crop (Barker and Mills, 2011). In addition, nitrogen nutrition influences both the primary and secondary metabolic pathways thus secondary plant metabolites accumulation (Chen *et al.* 2011) [54].
Most of the studies focused on the head production of artichoke as vegetable and there are limited studies about fertilization influence on artichoke growth and phytochemical compound. Moreover, given the fact that the use of excessive nitrogen fertilizer can be a waste of money

and leads to a problem for human health and environment. So the purpose of this research was to explore the effect of different levels of nitrogen fertilizer on morphological and phytochemical features of artichoke leaf and evaluation of its antibacterial activity and determine the appropriate nitrogen fertilizer for this plant.

Materials and Methods

Experimental site

This study was conducted in Isfahan (18 km west Isfahan, 32° 37' N, 51° 28'E and an altitude of 1612 m), Iran in 2014. Field has a semi-arid climate according to the Goshen climate classification. Annual average temperature and the annual average rainfall were 16 °C and 140 mm, respectively. The soil was a clay loam that its characteristics were as follows: organic matter 0.45%, total nitrogen 0.04%, available phosphorus 14 ppm and available potassium 250 ppm and pH 7.7.

Design type and analysis

A randomized complete block design with three replications was used in the study. Treatments were four levels of nitrogen fertilizer (0, 100, 150 and 200 kg.ha^{-1} pure nitrogen). The means of the data were compared by Duncan's multiple range test. Correlation between phytochemical parameters were analyzed using Pearson correlation analysis. All statistical procedures were performed by SPSS software.

Artichoke planting and management

Half dose of urea fertilizer was added to the soil before sowing time and the rest was applied when the artichoke plants presented eight leaves. Seeds were planted in April 2014. Experimental plot size was 5 m ×3.5 m and each plot consisted of 5 rows with adjacent rows being 70 cm apart. The distance between plants in each row was 35 cm. Then all plots were irrigated. Weed control was carried out by hand during growing season.

Evaluated traits

Some properties such as the leaf number per plant, leaf length, plant fresh weight and plant dry weight were investigated through randomly measuring 10 plants in each plot at vegetative rosette stage (November 2014). In laboratory total phenols, total flavonoids and antioxidant activity were measured.

Preparation of plant extracts

After harvesting, leaves were dried in the shade and ground into a fine powder. For preparing a methanolic leaf extract, a certain amount of ground leaf (20 g) was mixed with methanol 80% (200 ml) and filtered after 24 hours. The extraction was repeated three times for each sample and the methanol was evaporated using a rotary evaporator.

Determination of total phenolic content

Total phenol was measured by the Folin ciocalteau method. 0.5 ml of extract was mixed with 5 ml of Folin-Ciocalteau and 4 ml of sodium carbonate (1 M) and the absorption recorded at 760 nm after 20 minutes. Gallic acid was used as a standard for the calibration curve (McDonald *et al.*, 2001) [35].

Determination of total flavonoids content

Flavonoid content of the extracts was estimated according to Chang *et al.*, (2002) [13]. Plant extract (0.5 ml) was mixed with 1.5 ml of 80% methanol, 0.1 ml of aluminum chloride (10%), 0.1 ml of potassium acetate (1M) and 2.8 ml of distilled water. Quercetin was used for calibration curve and the absorbance of samples was read by spectrophotometer at 420 nm after 30 minutes incubation at room temperature.

Determination of antioxidant activity

Antioxidant activity was assessed using diphenylpicrilhydrazyl (DPPH) and reducing power methods.

DPPH free radical scavenging activity

Briefly, 1 ml extract was mixed with 2 ml DPPH (0.1 mM) and incubated in the dark for 30 min. The absorption of samples and control were read at 517 nm. Standard antioxidant (ascorbic acid) was used for comparison as positive control. DPPH free radical scavenging activity was calculated by using the following Equation (Krings and Berger, 2001) [31].

Scavenged DPPH radical (%) = (A control–A samples) / A control) × 100

Reducing power

Reducing power of methanolic extract was determined according to the method of Oyaizu (1986), 2.5 ml of extract from each sample in 2.5 ml of phosphate buffer (0.20 mol, pH 6.6) was added to 2.5 ml of potassium ferricyanide (10 mg/ml), mixture was incubated at 50°C for 20 min. 2.5 ml of trichloroacetic acid (100 mg/ml) was added to the mixture then centrifuged at 650 g for 10 minutes. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml ferric chloride solution (1 mg/ml) was added and the absorbance was recorded using a spectrophotometer at 700 nm. The higher absorbance of the reaction mixture indicated greater reducing power.

Results and Discussion

Number of leaves per plant

The number of leaves in the plant was significantly ($p \leq 0.01$) affected by the application of various amounts of nitrogen (Table 1). Nitrogen fertilization increased the number of leaves per plant. Table 2 showed that the maximum number of leaf per plant (30) was recorded by 200 kg N ha^{-1}. The minimum number of leaf per plant (16) was obtained in control plants (Table 3). The increase in the number of leaves per plant could possibly be ascribed to the fact that nitrogen often increases plant growth and subsequently more production of leaves. The improvement of vegetative characteristics with an increase in nitrogen fertilizer rate could be attributed to increased uptake of nitrogen and its associated role in chlorophyll synthesis and hence the process of photosynthesis and carbon dioxide assimilation (Jasso-chaverria *et al.*, 2005) [27] leading to enhanced growth. In addition, nitrogen stimulates vegetative growth. Also it mediates the utilization of potassium, phosphorus and other elements in plants and the optimum amounts of these elements in the soil cannot be utilized efficiency if nitrogen is deficient in plants (Brandy, 1984) [12]. Salamah (1997) [50] found that increasing N-fertilization from 95 to 285 kg N ha^{-1} significantly increased the number of artichoke leaves. Ng'etich *et al.*, (2013) [37] study the effect of different rates of nitrogen fertilizer (0, 40, 80, 120 and 160 kg N ha^{-1}) on the growth of Zucchini (*Cucurbita pepo* L.). They stated that plants subjected to 160 kg N ha^{-1} exhibited an increase of about 61.0 - 204.1% in the number of leaves compared to the control.

Leaf length

The effect of different doses of nitrogen fertilizer on the length leaf of artichoke was significant ($p \leq 0.01$) (Table 1). Application of 200 kg N ha^{-1} and control treatments had the highest and lowest rates of leaf length with 102 and 80.5 cm, respectively. The treatment of 100 and 150 kg N ha^{-1} were at the same statistical groups by 95 and 95.5 cm, respectively (Table 2). Hejazi *et al*. (2013) [22] reported that 250 kg ha^{-1} nitrogen manure and control treatments had the highest and lowest values of leaf length respectively. Dastagir and Hussain (2015) [15] stated that maximum leaf length in *Aloe vera* L. was obtained by urea and DAP fertilizers urea. Barandozi *et al*., (2011) [9] observed an increase in leaf length of Aloe vera under N and P fertilizers.

Plant fresh weight

The fresh weight of the plant was significantly ($p \leq 0.01$) influenced by nitrogen fertilizer (Table 1). Table 2 shows that increased levels of N fertilizer significantly increased plant fresh weight. The highest and the lowest plant fresh weight were obtained by application of 200 kg N ha^{-1} and control with 3494.33 g and 1214.67 g, respectively. Nitrogen is of vital importance for plant growth due to being a part of amino acid, protein, enzymes and chlorophyll molecule (Abbas and Fares, 2008) [1]. The increment in plant fresh weight may be attributed to a greater proliferation of root biomass resulting in the higher absorption of nutrients and water from the soil leading to the production of higher vegetative biomass (Hamblin, 1985) [21]. A nutrient solution contains at least 130 mg N L^{-1} and rates of 100 and 250 mg L^{-1} of P and K produced the best shoot fresh and dry weight in artichoke plant (Elia and Santamaria, 1994) [17]. The reduction of nitrogen application from 300 to 500 kg N ha^{-1} resulted in a reduction of the total biomass of artichoke (Pedreno *et al*. 1996) [45]. Alizadeh *et al*., (2010) [5] reported that fertilization of *Satureja hortensis* with 1500 mg fertilizer /plant increased fresh and dry weight compared with control treatment. Khaghani *et al*., (2012) [29] indicated that application of the chemical fertilizers (NPK) significantly affect the total fresh weight of chicory (*Cichorium intybus* L.). A progressive increase in fresh and dry weight of Mas cotek (*Ficus deltoidea* Jack) was observed with increasing nitrogen rate (Sheikh and Ishak, 2016) [52].

Plant dry weight

According to Table 1, different levels of nitrogen had a significant ($p \leq 0.01$) effect on plant dry weight. Application of N fertilizer significantly increased the dry weight of artichoke plant compared to control treatment. Consumption of 200 kg N ha^{-1} and control treatment had the highest (804.67 g) and the lowest (598.33 g) dry weight, respectively (Table 2). The increase in plant dry weight was due to the increase of N in the root zone as a result of chemical fertilizer application. Nitrogen is the important constituent of nucleotides, proteins, chlorophyll and enzymes, involves in various metabolic processes which have a direct impact on vegetative and reproductive phases of plants (Ibrahim *et al*., 2011; Zhang *et al*., 2014) [25, 26, 56]. Increasing nitrogen consumption from 95 to 285 kg N ha^{-1} increased the leaf fresh weight and leaf dry weight of artichoke (Salamah, 1997) [50]. Usage of different urea doses was caused a significant differences dry weight of total leaves in *Aloe vera* L. (Dastagir and Hussain, 2015) [15]. Hossain *et al*., (2007) [23] observed that N fertilizers significantly increased the dry weight of *Aloe indica* leaves. The highest values of fresh and dry weights in Dill (*Anethum graveolens* L.) were recorded by using 60 kg N/feddan (Said-Al Ahl *et al*., 2015) [48].

Total phenols

Different levels of nitrogen fertilizer had a significant ($p \leq 0.01$) effect on the phenol content of leaves (Table 1). Total phenol decreased with increasing nitrogen doses from 0 to 200 kg N ha^{-1}. Maximum total phenol content was 99.36 mg GAE g^{-1} DW that obtained by control treatment. The treatment of 100 and 150 kg N ha^{-1} were at the same statistical groups by 97.52 and 95.99 mg GAE g^{-1} DW, respectively. Also, there was a significant difference between 150 and 200 kg N ha^{-1}treatments (Table 2). The results indicated that total phenols had a positive significant correlation with flavonoid and antioxidant activity (Table 3). The pharmaceutical value of the artichoke leaves has been widely studied and the results confirmed the leaves as a rich source of polyphenols (Wang *et al*., 2003). Negative effects of nitrogen on phenol concentration can possibly be attributed to competition for phenylalanine, which can either be used in phenolic synthesis or be incorporated into protein synthetic pathways (Margna, 1977) [34]. In addition, nitrogen is efficient in improving the protein accumulation (Stewart *et al*., 2001; Jones *et al*., 2007) [28], and so phenolic levels are decreased for a certain amount of phenylalanine. The researchers presented different results in relation to the effect of fertilization on the phenolic content of plants. Anttonen *et al*. (2006) [6] who indicated reduced phenolic concentrations in strawberry fruits by increasing nitrogen fertilization. The individual nutrient nitrogen application decreased the total phenol of broccoli heads (Jones *et al*., 2007) [28]. Higher phenolic contents of Basil (*Ocimum basilicum* L.) observed when nutrient availability was limited at the lowest applied nitrogen treatment (Nguyen and Niemeyer, 2008) [38]. Cultivated *Ocimum gratissimum* (L) and *Gongronema latifolium* (Benth) were treated with NPK (15:15:15) inorganic fertilizer at 100 kg ha^{-1}, 200 kg ha^{-1}, 300 kg ha^{-1}, 400 kg ha^{-1} and 500 kg ha^{-1}. 500 kg ha^{-1} and 100 kg ha^{-1} treatment levels produced the highest amount of phenols in the leaves (Osuagwu and Edeoga, 2012) [41]. Biosynthesis of secondary plant metabolites is stimulated by nitrogen deficiency and total phenolic concentration of sweet basil (*Ocimum basilicum* L.) significantly increased in N-starved plants (Argyropoulou *et al*. 2015) [7].

Total flavonoids

The impact of different nitrogen rates on the production of total flavonoids in artichoke was significant ($p \leq 0.01$) (Table 1). The concentration of flavonoids declined by increasing the rate of nitrogen fertilizer. The highest and lowest concentration of flavonoids in the leaves were obtained in control treatment and 200 kg N ha^{-1} with 1.30 and 0.71 mg QE g^{-1} DW, respectively (Table 2). Total flavonoids showed a positive significant correlations with total phenol and antioxidant activity (Table 3). Phenylalanine is a precursor for the formation of flavonoids and the enhanced Phenylalanine would increase the production of flavonoids (Ranelletti *et al*., 1999) [46]. Nitrogen modulates the biosynthesis of secondary metabolites such as flavonoid compounds (Aires *et al*., 2006) [2]. Awad *et al*., (2002) [8] mentioned that the increase in flavonoids under low nitrogen fertilizer might be related to increase in phenylalanine availability due to restriction of protein synthesis under N deficiency conditions. Ibrahim *et al*., (2011) [25, 26,] stated that the high amount of nitrogen decreased the production of secondary metabolites in *Labisia*

pumila Blume due to reduced phenyl alanine lyase activity that was correlated with low C/N ratio, photosynthetic rates and total non-structural carbohydrate. Various results have been reported by the authors about the effect of fertilizer application on plant flavonoid content. Kolodziej and Winiarska (2010) [30] observed that irrigation and fertigation positively influence flavonoids content in artichoke plants. The higher amount of flavonoids was observed in the fertigation variant. Moor *et al*., (2005) [36] showed that fertilizer treatment caused reduced flavonoid content in plants. The individual nutrient nitrogen application decreased the concentration of flavonoids in broccoli heads (Jones *et al*., 2007) [28]. Wu *et al*., (2013) [55] reported that nitrogen application decreased the total flavonoid content of jujube (*Ziziphus jujube* Mill.). The highest values of total flavonoids in fennel (*Foeniculum vulgare* Mill.) were obtained when fennel plants were supplemented with 50% NPK + 50% organic fertilizer and bio fertilizer when compared with control treatment (Salama *et al*., 2015). The highest values of total flavonoid were resulted by plants fertilized with 60 kg N/fed., with or without bio-fertilizer (Said-Al Ahl *et al*., 2015) [48].

Antioxidant activity

DPPH free radical scavenging activity

The effect of different amounts of nitrogen fertilizer on the antioxidant activity of leaves was significant ($p \leq 0.01$) (Table 1). DPPH free radical scavenging activity of artichoke leaves significantly decreased when N fertilizer increased from 0 to 200 kg N ha^{-1}. The highest value of DPPH free radical scavenging activity was observed in control treatment with 90.96% and the treatment of 100 and 150 kg N ha^{-1} were grouped in the same statistical category (Table 2). Antioxidative activity of the artichoke extract had a significant positive relationship with total phenol and total flavonoids which implies a higher possibility that DPPH antioxidative capacity is as a result of a higher accumulation of these compounds (Table 3). While N is an essential nutrient element for crop growth and quality, little is known about the effect of N supply on the antioxidant activity of artichoke. The phenolic and flavonoid content are crucial factors in determining the antioxidant activity of this plant (Nouraei *et al*., 2016) [40]. Fertilizer effects on antioxidant activity in medicinal plants has also been reported. Nguyen and Niemeyer (2008) [38] showed at the highest nitrogen treatment level, basil exhibited significantly lower antioxidant activity than under limited nutrient growing conditions and manipulation of nitrogen fertilization levels may be an effective method to increase the expression of polyphenolic compounds in basil (*Ocimum basilicum* L.). Alizadeh *et al*., (2010) [5] indicated that the use of chemical fertilizer in *Satureja hortensis* increased the antioxidant activity in all treatments but not significant. Fertilization increased generally the concentration and accumulation of polyphenols and antioxidant activity of *Artemisia annua* L. (Luo *et al*., 2013). Antioxidant activity of jujube (*Ziziphus jujube* Mill.) could be manipulated through fertilizer management (Wu *et al*, 2013) [55]. Heavy nitrogen fertilization in *Chrysanthemum morifolium* decreased antioxidant activity of flowers (Liu *et al*., 2010).

Reducing power

Reducing power was significantly influenced by applied N rate ($P \leq 0.01$). Increasing nitrogen consumption from 0 to 200 kg N ha^{-1} decreased reducing power of artichoke leaf extract. The maximum amount of reducing power (2.29) was detected in the control treatment and the minimum value (1.97) was observed in using 200 kg N ha^{-1}. Application of 100 and 150 kg N ha^{-1} were grouped in the same statistical category (Table 2). A significantly positive correlation was found between reducing power value with total phenol, total flavonoids and DPPH radical scavenging activity (Table 3). High antioxidant activity in artichoke extract under low N levels might be due to high accumulation of total phenol and total flavonoids in the plant. The effects of nitrogen rate on antioxidant properties of plant had been reported. Biesiada *et al*., (2008) [11] showed that the antioxidant activity of lavender flowers was higher in treatment fertilized with nitrogen in dose of 50 kg N·ha^{-1} and decreased at the rates of 100 and 200 kg N·ha^{-1}. Also, the antioxidant activity of leaves increased with intensive nitrogen fertilization. Increasing nitrogen supply reduced FRAP value of Leaf mustard (*Brassica juncea* Coss) (Li *et al*., 2008) [32]. Stefanelli *et al*., (2011) [53] indicated that N applications of 1200 mg·L^{-1} or higher can result in reduced antioxidant capacity in red oak lettuce leaves. Ibrahim *et al*., (2011) [25, 26,] study the effect of four levels of nitrogen fertilization (0, 90, 180 and 270 kg N/ha) on *Labisia pumila Blume*. They found that this plant exhibited significantly lower antioxidant activities than those under limited N growing conditions.

Table 1: Analysis of variance for morphological traits and phytochemical properties of artichoke under different levels of nitrogen fertilizer

SOV	DF	Mean squares (MS)							
		Leaf length	Number of leaves per plant	Plant fresh weight	Plant dry weight	Total phenols	Total flavonoids	DPPH free radical scavenging activity	Reducing power
Replication		4.07 ^{ns}	9.08**	116939.52**	5206.02**	9.92 **	0.01**	3.29*	0.04**
fertilizer		245.76**	112.66**	2852900.91**	24193.85**	10.30**	0.19**	10.51**	0.05**
Error		3.57	0.75	6237.57	185.52	0.76	0.001	0.612	0.003

NS: no significant, *Means significant at level ($p \leq 0.05$) and **Means significant at level ($p \leq 0.01$)

Table 2: Effect of different levels of nitrogen fertilizer on morphological traits and phytochemical properties of artichoke

Nitrogen (kg ha^{-1})	Leaf length (cm)	Number of leaves per plant	Plant fresh weight (g)	Plant dry weight (g)	Total phenols (mg GAE g^{-1} DW)	Total flavonoids (mg QE g^{-1} DW)	DPPH free radical scavenging activity (%)	Reducing power
Control (0)	80.5 c	16 d	1214.67 d	598.33 d	99.36 a	1.30 a	90.96 a	2.29 a
100	95 b	24 c	2088 c	732.33 c	97.52 b	1.11 b	89.38 b	2.12 b
150	95.5 b	27 b	2791.17 b	766.83 b	95.99 bc	0.91 c	88.98 b	2.08 bc
200	102 a	30 a	3494.33 a	804.67 a	95.14 c	0.71 d	86.44 c	1.97 c

In each column, means with the similar letters are not significantly different at 5% level of probability using Duncan's test

Note: The DPPH free radical scavenging activity of the positive controls - ascorbic acid was 98.46%.

Table 3: Correlation analysis among phytochemical properties of methanolic extract of artichoke leaves

	Total phenols	Total flavonoids	DPPH free radical scavenging activity	Reducing power
Total phenols	1			
Total flavonoids	0.81**	1		
DPPH free radical scavenging activity	0.69*	0.91**	1	
Reducing power	0.79**	0.85**	0.84**	1

** Correlation is significant at the 0.01 level (2-tailed, $p<0.05$); * Correlation is significant at the 0.05 level (2-tailed, $p<0.05$).

Conclusion

Nitrogen application influenced the growth and phytochemical characteristics of artichoke plant. Increased application of urea fertilizer resulted in an increased vegetative growth of artichoke whereas the content of phenol, flavonoid and antioxidant activity decreased. The highest of leaf length, number of leaves per plant, plant fresh weight and dry weight was observed in 200 kg N ha^{-1}. The maximum amount of total phenol, total flavonoid, and antioxidant activity was observed in control treatment. A significant positive relationship was obtained between antioxidant ability (DPPH and reducing power) with total phenol and total flavonoid suggesting that an increase in the anti-oxidative activities in artichoke under low nitrogen fertilization could be related to higher contents of these compounds. Nitrogen is an essential element for artichoke growth and development, but nitrogen application at higher rates negatively affected the quality of artichoke leaves. So, it is suggested that no excess N application should be applied when cultivating artichoke for its medicinal use.

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