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Phyto-Chemical and Morphological Characters of *Calendula officinalis* as Affected by Micronutrients

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Abstract

In order to study the effect of foliar applications of micronutrients on yield, extraction oil production and chemical composition of pot marigold (*Calendula officinalis*), two experiments were conducted in the randomized complete block design with three replications in Shahrekord, Iran during two growing seasons 2014 and 2015. The objectives of this study were to determine the effects of foliar Fe^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} applications (0, 200 and 400 ppm) on flavonoid, phenols, carotenoids, extraction percentage, dry weight of the flower and the number of flowers of pot marigold. Results showed that foliar applications of Fe^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} had significant effects on fresh and dry flower yield, number of flowers, extraction oil percentage and flavonoids, phenols, carotenoids of pot marigold plants, so that the highest dry weight of flowers (11.9 g per plant), number of flowers per plant (33.5), extraction oil percentage (1) and flavonoids (31.2 mg/ 100 g F.W.), phenols (56.2 mg/ 100 g F.W.) and carotenoids (0.8 mg/ 100 g F.W.) content were obtained from the application of a combination of Fe^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} at 400 ppm concentration as compared to control in the second year. It seems that in climatic and edaphic conditions of Shahrekord, the best treatments for obtaining the highest extraction percentage and morphological characters of Calendula is 400 ppm of Fe, Zn, Cu and Mn.

Keywords: Extraction, GC/MS, Pot marigold, Nutrition, Phytochemical.

INTRODUCTION

Calendula, also known as pot marigold (*Calendula officinalis* L.), is an annual herb belonging to the Asteraceae family (Borghei *et al*., 2011). It is usually multi-stemmed with a strong tap root. The vegetative parts of the plant are mid green, whereas the stems are angular and covered in fine hairs. Pot marigold is cultivated for its flowers with a receptacle or flowers without receptacle (Borghei *et al*., 2011), which are used as a medicinal raw material. Flavonoids, carotenoids, and polyphenols are the active ingredients previously reported in pot marigold flowers (Bunghez and Ion, 2011). The most extraction of this plant is formed at full blooming (0.97%), and the least extraction (0.13%) before flowering (Sedghi *et al*., 2011). Few studies have examined the effect of iron, copper, zinc, and manganese fertil¬ity on the production and productivity of *Calendula*, and they were not related to the pharmacological effect of chamomile.

Iron (Fe) is important in cytochrome structure. The Fe (II) form is usually below the detection level in plants (Heidari *et al*., 2011; Schönherr *et al*., 2005). Copper is an essential micro-element in higher plants, as it occurs as a part of the prosthetic groups of several enzymes (Dadhich and Somani, 2007; Datta *et al*., 2011; Habib, 2012). Zinc is an important micronutrient associated with several enzymatic activities in all photosynthetic plants. It is necessary in vital enzymes and growth regulators (Babaeian *et al*., 2012a; Heidarian *et al*., 2011; Samia and Mohmoud, 2009). Manganese is involved in many biochemical functions, primarily acting as an activator of enzymes such as dehydrogenases and decarboxylases involved in respiration, amino acid and lignin synthesis, and hormone concentrations (Younis *et al.*, 2013; Babaeian *et al*., 2012b).

In alkaline soils, nutrient concentrations may not be sufficient and therefore, micro-nutrients are immobilized in these soils quickly so that the roots of the plants cannot absorb them from the soil and some nutrients do not transit to leaves. In these places, foliar application of micro-nutrients could solve this problem and reduce soil fertilizer loss (Dadhich and Somani, 2007). Foliar fertilization is a particularly useful technique that can be designed to meet plants' specific needs for one or more micro- or macro-nutrients, especially trace minerals, and to correct deficiencies, strengthen weak or damaged crops, accelerate growth, and grow better and healthier plants (Khalifa *et al.*, 2009). Although many studies have been performed on the effect of micronutrients on plants (Babaeian *et al.*, 2012a,b; Datta *et al.*, 2011; Galavi *et al*., 2012; Habib, 2012; Rawia *et al*., 2010), few studies have been focused on examining iron, copper, zinc and manganese fertility in pot marigold, and they are not specific to the medicinal use of this plant. So, this work was aimed at investigating the effects of iron, zinc, copper, and manganese foliar applications on reproductive, yield and phytochemical characters of pot marigold plants.

MATERIALS AND METHODS

Two field experiments were established in Shahrekord (50°56' E., 32°18' N.) in South-Western Iran in 2014 and 2015. The physical and chemical properties of soil are shown in Table 1. Experiments were arranged in a randomized complete block design with a factorial layout with three replications. All experiments were carried out in triplicate. Seeds of *C. officinalis* var. Qazvin were obtained from the Pakan Bazr Company, Isfahan, Iran. Sowing was conducted manually, plant spacing in each row was 5 cm, and two seeds were placed in the soil. At 3-5-leaf phase, plants were thinned out to a final row distance. Four micronutrients including Librel Fe-Lo, Librel Cu, Librel Zn, and Librel Mn were applied in these experiments. Librel Fe-Lo contains 13.2%

Table 1. Physical and chemical properties of the soil for the experiment.

Year	Clay (%)	Silt (%)	Sand (%)	N total (%)	K available (mg/kg)	P available (mg/kg)	Zn (mg /kg)	Mn (mg /kg)	Fe (mg/kg)	Cu (mg/kg)	O.C (%)	pH	E.C ($dS.m^{-1}$)	Depth (cm)
2014	39	33	28	0.158	741	15.9	0.54	0.99	3.1	1.1	1.13	8.05	0.76	0-30
2015	39	32	29	0.17	720	16.2	0.49	1.1	3.2	0.9	12	7.89	0.73	0-30

chelated iron, Librel Zn is a foliar fertilizer that contains 14% Zinc in chelated form, Librel Cu has 14% copper in chelated form, and Librel Mn is inclusive of 13% Mn chelated with EDTA (obtained from the Chemical Company of England and Germany). These fertilizers were sprayed at four concentrations (Fe1, Fe2, Fe3, and Fe4 were 0, 200, 400 and 600 ppm of Fe, respectively and similarly for the other elements (Zn, Cu and Mn) were considered).

Top soil of the experimental plot area was kept moist throughout the growing season when necessary. After the soil test, the required nutrients were added to soil. At the end of the blooming stage, pot marigold plants were harvested from middle rows of each plot. Several parameters including the number of the flowers, dry and fresh weight of the flowers, and the amount of essential oils percentage as well as the chemical components of the extraction (carotenoids, flavonoids and phenols) were determined.

Thermo Finnegan Trace 2000 GC/MS, made in the United State, was employed with an HP-5MS capillary column (30 m long and 0.25 mm wide, and a 0.25 µm of film thickness) at 250$^{\circ C}$ of the injector chamber. The initial column temperature was at 120$^{\circ C}$ for 5 min and then, increased to 280$^{\circ C}$ at the rate of 10$^{\circ C}$/min. Helium was used as a carrier gas at a rate of 35 ml/min. MS parameters were as follows: ionization energy, 70eV; ion source temperature, 200$^{\circ C}$; voltage, 3000 v; and mass range, 30 to 600. The compositions of the essential oil were identified by a comparison of their retention indexes, retention times, and mass spectra with those of authentic samples in Wiley library. Harvested flowers were dried at room temperature for a week. Flower extracts were obtained by stirring 1 g of dry flower powder with 10 ml of pure methanol for 30 min. The extracts were, then, kept for 24 h at 4$^{\circ C}$, filtered through a Whatman no. 4 filter paper, evaporated under vacuum to dryness, and stored at 4$^{\circ C}$ until they were analyzed. The total phenolic content of *C.officinalis* methanolic extract was determined using the Folin–Ciocalteu (F–C) reagent (Adams, 2001).

Dried samples were hydrolyzed, and slightly modified. Twenty ml of methanol containing BHT (1 g l^{-1}) was added to 0.5 g of a dried sample. Then, 10 ml of 1M HCl was added. The mixture was stirred carefully, sonicated for 15 min and refluxed in a water bath at 90$^{\circ C}$ for 2 h. The obtained mixture was injected to HPLC. The phenolic compounds analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph (RP-HPLC, Palo Alto, CA) coupled with a UV–vis multiwavelength detector. The separation was carried out on a 250 × 4.6-mm, 4µm Hypersil ODS C18 reversed-phase column at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water with 0.2% sulfuric acid (solvent B). The flow rate was kept at 0.5 ml min^{-1}. The gradient program was as follows: 15% A/85% B, 0–12min; 40% A/60% B, 12–14min; 60% A/40% B, 14–18min; 80% A/20% B, 18–20 min; 90% A/10% B, 20–24min; and 100% A, 24–28 min. The injection volume was 20 µl, and peaks were monitored at 280 nm. Samples were filtered through a 0.45 µm membrane filter before the injection. The peaks were identified by congruent retention times compared with standards. Analyses were performed in triplicate (Bourgou *et al.*, 2008).

All data were submitted to ANOVA by using the statistical computer package SAS ver. 8. When the treatment effects were significant ($P<0.05$) as compared to control, treatment means were separated using Least Significant Difference test at the significance level ($P<0.05$).

RESULTS

There were significant differences between treatments (Table 2). Although there were no significant differences in some of the single treatments, in the combinations of treatments, there were differences in most characters that resulted in the $Fe_3Cu_3Mn_3Zn_3$ treatment being the best (Tables 2-4). It seems that synergist application of micronutrient results in the additive effectiveness of measured characters, but the fertilizer tended to be toxic at rates higher than 400 ppm. The components of *Calendula* oil were influenced by different treatments. The application of the Fe, Cu, Mn, and Zn significantly improved the growth characters, including the number of the flowers, dry weight of the flowers, essential oil percentages as well as the chemical component of the extraction (carotenoids, flavonoids and phenols); these factors showed a significant response to the

Table 2. Complex analysis of variance of variation of flavonoids, phenols, carotenoids, extraction percentage, fresh weight of flower, flower dry matter, number of flowers, and root/shoot dry/fresh matter in plants of *Calendula officinalis* L.

S.o.V	df	Flavonoid	Phenols	Carotenoids	Extraction percentage	Dry weight of flower	Number of flowers
		Mean of Squares					
Year	1	0.002*	0.01*	0.043*	0.016*	4.4	5.1*
R/Y	4	0.003*	0.018*	0.051*	0.018*	6.7*	4.9*
A (Cu, Zn, Mn,Fe)	3	0.0022*	0.014*	0.024*	0.0018*	8.5*	5.4*
B (0. 200. 400,600)	3	0.0037**	0.03**	0.05**	0.03**	14.4**	8.6**
A× B	9	0.0016*	0.09*	0.017*	0.0122*	5.95*	3.77*
T(A, B)Y	15	0.00139**	0.082**	0.0161*	0.012*	5.43*	3.52*
A×Y	3	0.0021*	0.0129*	0.032*	0.0019*	8.8*	5.1*
B×Y	3	0.0019*	0.014*	0.028*	0.002*	9.1*	4.8*
	9	0.0009	0.127**	0.0068	0.018**	3.1	2.57
	60	0.0007	0.0041	0.0078	0.0055	2.7	1.7
		4.2	2.4	3.3	1.2	8.7	6.8

*and**: Significant at P<0.05 and P<0.01 levels of probability, respectively.

increasing rates of foliar fertilizers (Table 2).

Meanwhile, the effect of a combination of the four micronutrients surpassed that of each of them alone. Thus, the foliar application of 400 ppm Fe, Cu, Mn, and Zn caused the highest increments as compared to other treatments. In case of the number of flowers per plant, control plants and plants treated with 600 ppm of micronutrients exhibited similar behaviors. The application of 400 ppm Fe, Cu, Mn and Zn increased flavonoids, phenols and carotenoids in the essential oil of calendula. However, a combination of $Fe_3Cu_3Mn_3Zn_3$ treatment was more effective than other treatments. Most meas-

Table 3. Results of correlation between characters in *Calendula* plants that are affected by several micronutrients in first year.

Characters	Number of flowers	Flower dry weight	Extraction percentage	Carotenoids	Phenols	Flavonoid
Number of flowers	1					
Flower dry weight	0.9**	1				
Extraction percentage	0.8**	0.7**	1			
Carotenoids	0.8**	0.68**	0.5	1		
Phenols	0.6**	0.8**	0.46	0.5	1	
Flavonoids	0.65**	0.56**	0.69**	0.69**	0.65**	1

ns: Not significant, *and** : Significant at P<0.05 and P<0.01 levels of probability, respectively.

Table 3. Continued- Results of correlation between characters in *Calendula* plants that are affected by several micronutrients in the second year.

Characters	Number of flowers	Flower dry weight	Extraction percentage	Carotenoids	Phenols	Flavonoid
Number of flowers	1					
Flower dry weight	0.79**	1				
Extraction percentage	0.68**	0.87**	1			
Carotenoids	0.78**	0.88**	0.5	1		
Phenols	0.6**	0.78**	0.55*	0.5	1	
Flavonoids	0.65**	0.76**	0.9**	0.59*	0.6**	1

ns: Not significant, *and** : Significant at P<0.05 and P<0.01 levels of probability, respectively.

ured traits (for example, number and weight of flowers) were decreased with Fe, Cu, Mn, and Zn rates of higher than 400 ppm. The application of over-optimal range of micronutrients decreased the yield and extraction production. Control plants showed higher yield components than 600 ppm Fe, Cu, Mn, and Zn. In most treatments, $Fe_3Cu_3Mn_3B_3$, $Fe_3Cu_3Mn_2Zn_2$ and $Fe_3Cu_3Mn_1Zn_1$ mixtures made the maximum amount of the traits, but $Fe_3Cu_3Mn_3B_3$ was found to be the best combination (Tables 3, 4). In most cases, control plants showed similar behavior as $Fe_4Cu_4Mn_2Zn_2$, $Fe_4Cu_4Mn_3Zn_3$ and $Fe_4Cu_4Mn_4Zn_4$. Similar results were obtained from control plants for toxicities of higher concentrations of Fe, Cu, Mn, and Zn. It is obvious from the presented data that the highest levels of the four

Table 4. Means of characters measured in *Calendula* plants that are affected by micronutrients (400 ppm) concentration and control plants (1st year).

Characters	Number of flowers	Weight of flower (g/plant)	Extraction percentage	Phenols (mg/ 100 g F.W.)	Flavonoid (mg/ 100 g F.W.)	Carotenoids (mg/ 100 g F.W.)
Copper (Cu)	21.1 de	7.0 cd	0. 24 d	28.4 d	14.1 c	0.07 de
Manganese (Mn)	22.1 d	7.5 cd	0. 22 d	28.2 d	14.2 c	0.11 d
Iron (Fe)	22.2 d	8.0 c	0. 25 d	32.9 d	14.4 c	0.09 d
Zinc (Zn)	20.1 de	6.6 d	0. 24 d	30.1 d	15.1 c	0.10 d
Cu×Mn	22.4 cd	9.1 b	0. 56 c	39.1 bc	15.5 c	0.23 bc
Cu×Fe	23.1 cd	9.3 b	0. 52 c	38.4 bc	16.2 c	0.22 bc
Cu×Zn	24.4 cd	9.4 b	0. 55 c	37.0 c	16.7 bc	0.18 bc
Mn×Fe	25.7 c	9.5 b	0. 59 bc	38.8 bc	19.5 b	0.17 bc
Mn×Zn	23.1 cd	9.5 b	0.54 c	38.1 c	18.5 b	0.15 c
Fe×Zn	25.5 c	9.6 ab	0. 62 bc	40.2 bc	18.0 bc	0.25 bc
Cu×Mn×Fe	27.2 b	10.2 a	0. 71 b	44.1 b	20.1 b	0.30 b
Cu×Mn×Zn	27.4 b	10.4 a	0. 7 b	42.2 b	22.0 b	0.38 b
Cu×Fe×Zn	27.6 b	10.4 a	0. 8 ab	44.0 b	24.0 ab	0.39 b
Fe×Zn×Mn	27.1 b	10.1 a	0. 8 ab	43.0 b	23.0 b	0.40 b
Cu×Zn×Mn×Fe	33.1 a	10.8 a	0.9 a	55.3 a	30.0 a	0.60 a
Control	18.1 e	6.9 d	0. 21 d	25.1 d	12.5 c	0.05 e

*Similar letter(s) in each column show insignificant differences at the 1 and 5% probability level according to the LSD test.

Table 4. Continued- Means of characters measured in *Calendula* plants that are affected by micronutrients (400 ppm) concentration and control plants (2nd year).

Characters	Number of flowers	Weight of flower (g/plant)	Extraction percentage	Phenols (mg/ 100 g F.W.)	Flavonoid (mg/ 100 g F.W.)	Carotenoids (mg/ 100 g F.W.)
Copper (Cu)	22.1 cd	8.0 d	0. 3 de	28.6 d	15.2 d	0.07 de
Manganese (Mn)	23.1 cd	8.1 d	0. 31 de	28.4 d	15.3 d	0.11 d
Iron (Fe)	23.2 cd	9.0 c	0. 34 de	33.1 cd	14.7 d	0.09 d
Zinc (Zn)	21.1 d	7.0 d	0. 27 e	31.1 d	15.5 d	0.07 de
Cu×Mn	23.4 cd	10.1 b	0. 57 c	39.5 bc	15.6 d	0.23 bc
Cu×Fe	24.1 c	10.2 b	0. 55 c	38.6 c	16.5 d	0.22 bc
Cu×Zn	24.2 c	10.0 b	0. 56 c	37.5 c	16.9 cd	0.20 c
Mn×Fe	26.4 bc	10.2 b	0. 6 c	39.8 c	20.1 c	0.20 c
Mn×Zn	25.1 bc	10.1 b	0.57 c	38.5 c	19.9 c	0.20 c
Fe×Zn	26.5 bc	10.2 b	0. 64 c	41.2 bc	18.8 c	0.35 bc
Cu×Mn×Fe	28.2 b	11.7 a	0. 74 b	45.1 b	21.5 bc	0.35 bc
Cu×Mn×Zn	28.4 b	11.6 a	0. 73 b	43.2 bc	22.3 bc	0.40 b
Cu×Fe×Zn	28.6 b	11.1 ab	0. 82 b	45.2 b	24.5 b	0.40 b
Fe×Zn×Mn	28.1 b	11.7 a	0. 9 ab	45.1 b	23.4 b	0.41 b
Cu×Zn×Mn×Fe	33.5 a	11.9 a	1.0 a	56.2 a	31.2 a	0.80 a
Control	18.3 d	7.1 d	0. 22 e	26.1 d	14.2 d	0.05 e

*Similar letter(s) in each column show insignificant differences at the 1 and 5% probability level according to the LSD test.

foliar fertilizers were more effective than the lower levels, and that Librel Fe-Lo fertilizer was superior to other micronutrients. However, the highest essential oil percentage was found with $Fe_3Cu_3Mn_3Zn_3$. The concentration of micronutrients positively affected the measured characters and between the number of flowers, dry weight of flowers and the amount of essential oils percentage as well as the chemical component of the extraction were determined of plants were positive correlated and in best combinations of treatments (Tables 3, 4) the $Fe_3Cu_3Mn_3Zn_3$ was the best. Essential percentage positively correlated with the number, and weight of flowers of pot marigold plants (Tables 3 and 4).

DISCUSSION

Results showed that micronutrient is beneficial to pot marigold plants (Table 1). Higher micronutrients can result in higher yield, extraction and essential oils from *Calendula* (Naguib *et al.*, 2005). The beneficial application of micronutrients was reported by other researchers (Galavi *et al.*, 2012; Yadegari, 2015). Results of the present research showed that foliar application of micronutrients improved essential oils in the flowers of pot marigold plants (Tables 3, 4). A similar effect of micronutrient supply was also reported in *M. chamomilla* (Nasiri *et al.*, 2010), *Coriandrum sativum* (Said-Al Ahl and Omer, 2009), *Mentha* sp. (Rawia *et al.*, 2010), and *Ocimum basilicum* (Said-Al Ahl and Mahmoud, 2010). In this study, the effect of Fe^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} applications was determined on the growth and yield of pot marigold plants in two consecutive years.

Dry matter flower was increased in both years with micronutrients applications. The essential oils yield was increased with Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} applications because there was a significant increase in dry matter and the number of flowers. This is the first report in which the effects of Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} applications were determined on certain physiological characteristics for pot marigold plants and also their relationship with yield and essential oil content. It is obvious that weight and the number of flowers can be used as a tool for selecting new cultivars with higher yield. It is concluded from the present data that applications of Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} are beneficial for plants at the concentrations of 400 ppm or lower, and can result in an increase in dry matter yield of approximately 40%.

Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} have direct functions on the growth and development of plants. There are still many unanswered questions about how Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} act in increasing yield and its components (number of flower plants) for pot marigold plants. One possibility is that the foliarly applied Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} can affect dry matter accumulation and increase the dry matter flower. It seems that control plants, which had no foliar application, were better than plants that received higher concentration of micronutrients. Combinations of the micronutrients at higher than 400 ppm concentration had greater reducing effect than their application alone. These results reflect the role of applying the four foliar fertilizers in improving the total essential oils in pot marigold plants. It seems that Fe, Cu, Mn, and Zn made a change in metabolism, growth, and development and then increased upper phytochemicals by affecting the absorption and transition of essential nutrients.

Generally, the obtained results revealed that applying foliar fertilizers such as Fe, Cu, Mn, and Zn at 400 ppm resulted in the highest improvement of growth character, yield, and chemical constituents.

CONCLUSION

Plants treated with 400 ppm of iron, zinc, manganese, and copper resulted in higher fresh and dry weight of the flowers and extraction percentage. It could be concluded from the results that iron, zinc, manganese, and copper fertilization had a significant effect on the number of flowers, dry flower matter, and the amount of extraction percentage as well as the chemical component of the extraction of pot marigold plants. The highest number of flowers per plant, weight of flowers per plant, and extraction percentage were obtained from the plants treated with 400 ppm of iron, zinc, manganese, and copper. The most suitable Fe-Zn-Mn-Cu supply for production of pot marigold plants with the highest number and weight of flowers in Shahrekord, Iran is 400 ppm foliar application of micronutrients. The effect of combined application of micronutrients is suggested in comparison with their alone use.

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