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Effect of Exogenous Nitric Oxide Application on Secondary Metabolite Content of Marigold (*Calendula officinalis* L.)

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ABSTRACT: Marigold (*Calendula officinalis* L.) is an important herbal plant because of its phenolic compound. Nitric oxide which is a signal molecule could impressed development processes and secondary metabolite production in plant. The effect of spraying three level of sodium nitroprussiate (SNP) as a NO donor was studied on content of phenolic compound and some other property and growth indices of *Calendula officinalis* L. The data of experiment shown that SNP treatment probably have significantly roll in production of phenolic compound, flavonoid content, antioxidant activity and essential oil of plant capitule, but not effective on other pigment content and some growth indices like flower dry weight.

Key words: *Calendula officinalis* L., nitric oxide, phenolic content, flavonoid, antioxidant.

INTRODUCTION

Marigold (*Calendula officinalis* L.) is a beautiful annual plant of the Asteraceae family with yellow and orange flowers that are of great importance in green space and ornamental gardens. Moreover, this plant has numerous medicinal and healthcare applications (zu Beerentrup and Röbbelen 1987).

The most important medicinal use of pot marigold is in preparation of anti-inflammatory drugs for mild burns and of skin ointments. Its extract has antimicrobial and antifungal properties, and there are reports that it also has antiviral properties.

The active ingredients in this plant include a wide group of flavonoid compounds, though it also contains substantial quantities of monoterpenes, sesquiterpenes, and essential oil (Yoshikawa *et al*. 2001).

Most of these compounds are in capitule, especially in petals (Wyk and Wink 2004); and the flowers are rich in carotenoids. Compounds in pot marigold petals have strong antioxidant properties and enjoy considerable potential in helping to repair damages to human skin (Fonseca *et al*. 2010).

Nitric oxide (NO) is a gaseous hydrophobic substance that plays various roles in eukaryotes. In plants, NO controls a wide spectrum of growth and development processes (Wang *et al*. 2015). Many physiologists believe that the main duty of NO is to transmit biosignals or, more precisely, that NO molecules act as secondary messengers in cells. That is why the response spectrum created in plants in treatments containing this compound is very broad and efficient (Durner and Klessig 1999). Most responses are related to enhancing defense mechanisms against ROS. PAL is the first enzyme in the phenylpropanoid pathway, and has the important duty of linking primary metabolism to secondary metabolism and of producing secondary metabolites (Dixon and Paiva 1995). Under non-oxidative deamination conditions, PAL converts phenylalanine into trans-cinnamate (Boudet 2007; Vogt 2010), from which many plant phenolic compounds originate. Put more simply, it has been found that, in many cases, more plant phenolic compounds are produced with increased PAL activity (Wang and Wu 2005).

It has been clearly demonstrated that NO stimulates transcription of the PAL gene in plants (Durner, Wendehenne, and Klessig 1998). Increased PAL production means greater efficiency in converting phenylalanine into phenolic compounds; and, therefore, in most cases concentrations of plant phenolic compounds increase following the use of NO.

In ginger (*Zingiber officinale*), solutions of SNP (the compound that releases NO) increased resistance to chilling stress (Li, Gong, and Xu 2014). Spraying wheat leaves with NO resulted in greater resistance to stress in addition to increasing plant fresh weight (Kausar and Shahbaz 2013). Soaking seeds (priming) with NO releasing compounds such as SNP is one of the practical strategies in using NO. For example, soaking fenugreek seeds in SNP solutions for 24 hours increased the antioxidant activities of this plant (Gupta and Mandal 2016).

NO also activated defense systems of plants and increased production of secondary metabolites even under stress-free conditions. Priming fenugreek seeds with SNP solutions increased the content of phenolic and flavonoid compounds in the plants (Gupta and Mandal 2016). Priming of ripe litchi fruits with SNP solutions increased the total content of phenolic compounds during their storage life (Barman *et al*. 2014). Treating lemon balm (*Melissa officinalis*) seedlings under in vitro conditions increased the total content of phenolic and flavonoid compounds in the plants.

There is little information available regarding the effects of NO on production of phenolic compounds in pot marigold, and production efficiency of this plant will improve with increases in its content of phenolic compounds. Therefore, this experiment was conducted to evaluate the effects of treating seeds and mature plants of pot marigold on their crop production and on production of secondary metabolites, and to compare these two methods with each other.

MATERIAL AND METHOD

A. Sample collection

In this study nitric oxide Hormone was sprayed on the leaves in the summer. At the end of season, total plant mass was collected from and immediately transported to the horticulture laboratory at the University of Tabriz, Iran.

B. Total phenolic and flavonoid contents

In this study, the total phenolic and flavonoid contents of methanol extracts were measured according to the methods described by Abdel-Hameed (2009). TPC of plant extracts was determined using Folin Ciocalteu's reagent. For this purpose100 µl of each sample solution (100 µg/ml) and also 100 µl of gallic acid (100 µg/ml) were mixed with 500 µl of the reagent and 1.5 ml of 20% sodium carbonate. The mixture was shaken and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 h. Then the absorbance at 765 nm was determined against a blank that contained all reagents without the samples or the gallic acid at the same conditions. All determinations were carried out in triplicates. The total phenolic content was expressed as the number of equivalents of gallic acid (GAE). The flavonoids content was determined by $AlCl_3$ method using quercetin as a reference compound. 100 µl of each sample solution (1 mg/ml) was mixed with 100 µl of 2% $AlCl_3$ in ethanol and a drop of acetic acid, and then diluted with ethanol to 5 ml. The absorption at 415 nm was read after 40 min. Blank was prepared from all reagents without the samples.

C. DPPH radical scavenging activity of leaf extracts

Antioxidant activity of the sample extracts was quantified by measuring the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The DPPH scavenging activity of the extracts was determined using spectrophotometry which was adopted from the method of Perera *et al*. (2013).

D. Malondialdehyde (MDA) content

Peroxidation of lipids was determined in terms of TBARS concentration as described by Noreen &Ashraf (2009). Fresh leaf (1g) was homogenized in 3 mL of 1% (w/v) TCA at 4°C. Homogenates were centrifuged at 13,000 rpm for 10 min and 0.5 mL aliquot of the supernatant was added to 3 mL of 0.5%(v/v) thiobarbituric acid in 20% TCA. The mixture was incubated at 95°C in a shaking water bath for 1h, and the reaction was stopped by cooling the tubes on ice. The supernatant were centrifuged at 11,000 rpm for 15 min, and the abs of the samples was determined at 532nm. The value for nonspecific absorption at 600 nm was subtracted. Absorption coefficient used for calculating of concentration of TBARS was 155mmol^{-1} cm^{-1}.

RESULTS AND DISCUSSION

A. Total Phenolic content

The experiment showed that - compared to the control - various concentrations of NO (nitric acid) included 50, 100 and 200 mM had significantly increased the total phenolic compounds.

The flower treated by applying SNP at concentration 200 mM exhibited a greater increase in phenolic compounds as compared with the other concentrations. In spite of this, 100 mM of SNP concentrations also exhibited significant differences in the content of phenolic compounds compared to the control (Fig. 1).

In many other plants, NO similarly increased phenolic compounds under various conditions. For example, application of NO in fenugreek increased phenolic compounds significantly. This has numerous health benefits for humans because phenlic compounds have substantial antioxidant properties (Gupta and Mandal 2016). Treatment of Chinese jujube with NO also increased the total phenolic compounds and improved PAL activity (Zhu, Sun, and Zhou 2009).

Considerable reduction in phenolic compounds was observed during the storage life of the longan fruit, but this trend slowed down substantially with the application of NO (Duan *et al*. 2007). Another research reported that application of NO on tomato plants under salt stress had increased their phenolic compounds (Ali and Ismail 2014). Furthermore, NO treatment in packaged button mushrooms (MDA) increased their total phenolic compounds, and some reports indicated that NO could increase PAL activity (Jiang *et al.* 2011). In addition, it seems increased PAL activity improves production of secondary metabolites such as phenolic compounds in plants. For example, NO application had a positive effect on PAL activity in a Taxus cell suspension treated with ultrasound (Wang and Wu 2005).

It was stated in another report that treatment of millet seeds with NO considerably improved PAL activity (Manjunatha *et al.* 2008). It was also found in other studies that NO application increased antioxidant properties of plants, and it was thought that this in turn caused increases in a series of metabolites like polyphenols and flavonoids.

Therefore, considering the increase in phenolic compounds with the application of NO in the present experiment, it seemed increasing PAL activity had increased concentration of phenolic compounds in the plants.

B. Flavonoid content of plant capitule

As shown in Fig. 1, increasing concentration of SNP increases the level of phenolic compounds, and subsequently, flavonoids in plant capitule. The best results were obtained when SNP was applied at 200 mM concentration, since it led to the production 293.75 $mg.kg^{-1}$ of flavonoids. In all concentration of SNP, flavonoid content were increased compared to control but no significant difference were noticed between three concentrations of SNP (Fig. 2).

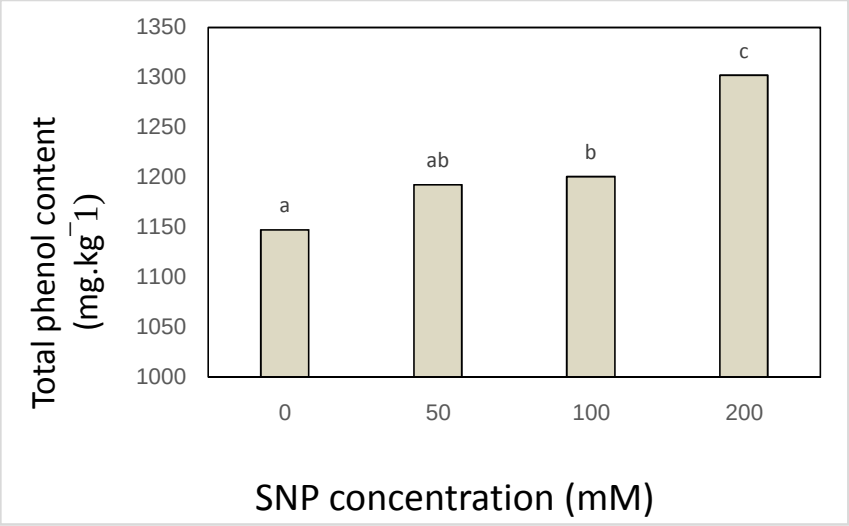

Fig. 1. Total phenolic content of *Calendula officinalis L.*Capitule induced by different SNP concentration. Data are means of 4 replicates. Different letters indicate significant differences between treatment according to Compare Means*Test– Duncan* ($p \leq 0.05$).

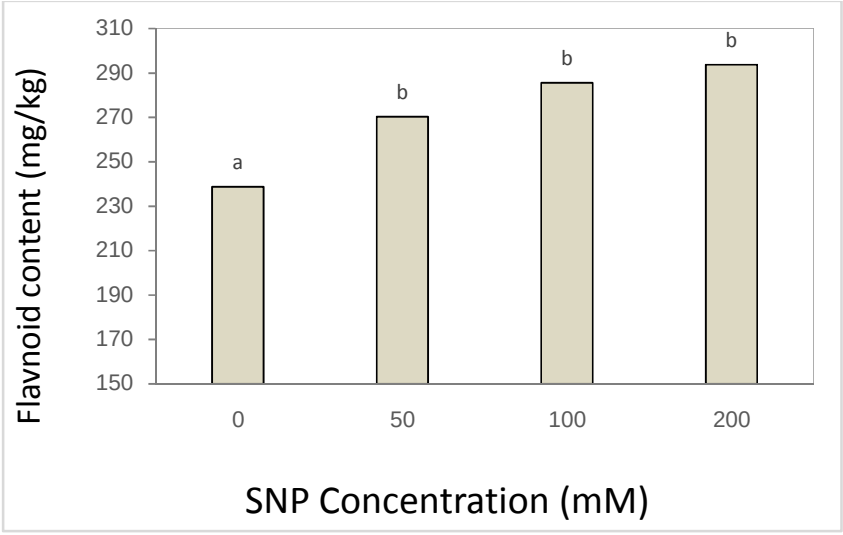

Fig. 2. Flavonoid content of *Calendula officinalis L.*Capitule induced by different SNP concentration. Data are means of 4 replicates. Different letters indicate significant differences between treatment according to Compare Means*Test– Duncan* ($p \leq 0.05$).

Flavonoids are classified as secondary metabolites and phenolic compounds(Heim, Tagliaferro, and Bobilya 2002), and they can protect plants against the harmful effects of UV rays, herbivores, and pathogens(Harborne and Williams 2000). They also have beneficial effects for human health, probably due to their antioxidant and chelating properties (Heim, Tagliaferro, and Bobilya 2002).

A series of studies reported that various concentrations of SNP had increased flavonoids in plants. For example, NO application in ginger increased synthesis of flavonoids (Li, Gong, and Xu 2014). It was stated in a report that application of NO had increased flavonoid content of fenugreek by 50% as compared to the control (Gupta and Mandal 2016).

Exogenous NO in a Ginkgo cell suspension excited by UV-B reportedly increased biosynthesis of flavonoids (Hao *et al.* 2009).

Since flavonoids are classified as phenolic compounds, and since - in our report - both these compounds exhibited an ascending trend, it seems the increase in both these cases resulted from changes in PAL activity.

C. Pigment content of plant capitule

Carotenoids, chlorophyll A, chlorophyll B and total chlorophyll contents at three levels of SNP application were measured in the capitule of the studied plants. The obtained results, listed in Table 1, showed that SNP application did not effect on chlorophyll A and B contents or total chlorophyll cantent in the plants, and also had no significant effect on carotenoid content.

Table 1: Some pigment content of calendula capitule induced by different concentration of SNP. Data are means of 4 replicates. Different letters indicate significant differences between treatment according to Compare Means *Test*– *Duncan* ($p \leq 0.05$).

Chlorophyll a ($mg.kg^{-1}$)	Chlorophyll b ($mg.kg^{-1}$)	Carotenoid ($mg.kg^{-1}$)	Total chlorophyll ($mg.kg^{-1}$)	SNP concentration (mM)
0.54 b	0.705 c	215.75 d	1.245 a	0
0.55 b	0.715 c	218.8 d	1.2775 a	10
0.6225 b	0.7375 c	224.95 d	1.325 a	20
0.6325 b	0.78 c	230.075 d	1.41 a	30

Leaf chlorophyll content provides us with information about the physiological condition of the plant. Many enzymes are involved in the disintegration of chlorophyll, and NO application probably suppresses a series of these destructive enzymes and leads to increased levels of this pigment in plants (Shi *et al.* 2016). Some of experiment have shown that exogenous NO application could increase content of pigment in plant under stress condition, but had no significant affect when the plant was not in any stress condition.

It seems pretreatment of broccoli with SNP significantly reduces the enzyme levels that disintegrate chlorophyll, including Chl-POX and PPH, which is the reason for the increased chlorophyll A and B levels in the florets of this plant (Shi *et al.* 2016). Furthermore, it was stated in another report that NO led to considerable reduction in chlorophyll destruction of sunflower leaves when they were exposed to Cd toxicity, but could not increase chlorophyll content in non-stress condition (Laspina *et al.* 2005). In another experiment, spraying SNP on Lilium leaves increased leaf chlorophyll A and B contents under non-stress conditions. This study also reported that NO increased plant chlorophyll content under stress and non-stress conditions (Wang *et al.* 2015). Application of NO on banana fruit also increased fruit chlorophyll A and B contents, which resulted from decreased activity of chlorophyllase (an enzyme that restricts chlorophyll catabolism) (Wang, Luo, and Du 2015). Application of NO to chickpea plants increased chlorophyll A and B contents under normal conditions, but carotenoid content in this case increased only under salt-stress conditions (Ahmad *et al.* 2016).

Some of these results are similar to those stated in this recent report which NO had no significant effect on chlorophyll or carotenoid content of studied plant.

D. Growth indices

Dry weights and fresh weights of plants treated with three levels of SNP were measured. The obtained results suggest that SNP had no significant effect on growth indices of plants (Fig. 3).

It was stated in a report that pretreatment with NO increased plant dry weight under salt stress and drought stress conditions. In sunflower plants exposed to Cd toxicity, NO application also increased dry weight and raised it to the level observed before plants were exposed to Cd toxicity. Moreover, dry weight increased when SNP treatment was applied without toxicity, but not as much as the previous case (Laspina *et al.* 2005). Furthermore, NO application on maize seedling under salt stress conditions increased dry weight, but under non-stress condition, NO could not effect significantly on dry weight (Zhang *et al.* 2006). In another experiment, NO application on tomato seedling under copper stress, had no significant effect on root and shoot fresh weight.

E. Total antioxidant activity

Total antioxidant activity at three levels of SNP application was measured both in the essential oil and the capitule extract of the plants. The obtained results indicated SNP had increased total antioxidant activity of the plant. The antioxidant property of the capitule was determined in terms of their free radicals scavenging potential such as DPPH.

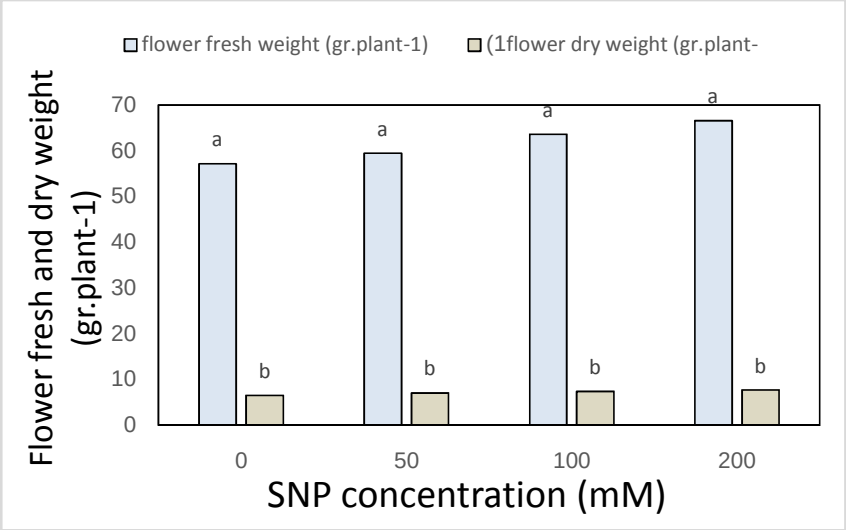

Fig. 3. Different concentration of SNP have no significant effect on some growth indices of *Calendula officinalis L.* Data are means of 4 replicates. Different letters indicate significant differences between treatment according to Compare Means*Test– Duncan* ($p \leq 0.05$).

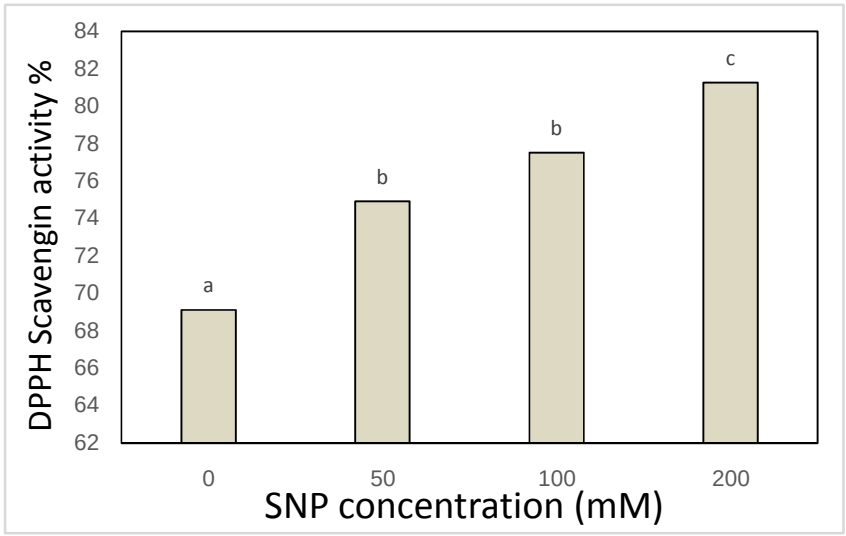

Fig. 4. Effect of different concentration of SNP on DPPH scavenging activity. Data are means of 4 replicates. Different letters indicate significant differences between treatment according to Compare Means*Test– Duncan* ($p \leq 0.05$).

Data have shown that each concentration of SNP significantly increased DPPH scavenging activity (Fig. 4). It appears exogenous NO can stimulate a series of antioxidant metabolites and thus increase total antioxidant activity (Ksouri *et al.* 2007). It is necessary to evaluate the plant extract antioxidant potential in order to determine its nutritional value (Rice-Evans, Miller, and Paganga 1996).

Treatment of ginger plants under conditions of cold stress with NO increased the activities of MDHAR, DHAR, APX, GPX, CAT, SOD, and GR. However, when the plants were not under stress, NO could increase only the activities of APX, SOD, and GR. It was reported that exogenous NO in SNP form could increase activity of antioxidant enzymes and protect the plants against oxidative damages (Li *et al.*, 2014).

Phenolic compounds having antioxidant properties can prevent a series of oxidative stresses (Wang *et al.* 2010). Various bioactive compounds such as phenols and anthocyanins control the antioxidant capacity in fruits.

Application of NO on Litchi plants considerably reduced antioxidant capacity reduction rate in the stored litchi fruit (Barman *et al.* 2014).

F. Essential oil

Essential oil contents of plant capitule at three levels of treatment with SNP were 0.1575%, 0.2075%, and 0.2275% respectively, which showed SNP had increased essential oil content (Fig. 5).

It was stated in a report that application of fungal cell suspension on Atractylodeslancea had increased its essential oil content. It seems NO, acting as a signaling molecule to mediate the AL4 elicitor, caused accumulation of the essential oil in the cell suspension of this fungus(Fang, Dai, and Wang 2009).

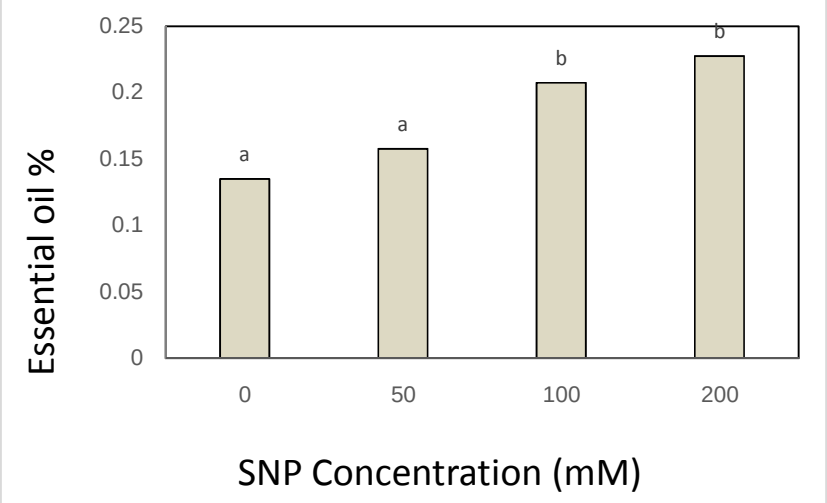

Fig. 5. Content of plant capitule Essential oil induced by different SNP concentration. Data are means of 4 replicates. Different letters indicate significant differences between treatment according to Compare Means*Test– Duncan* ($p \leq 0.05$).

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