



Effects of Silver Nanoparticle Exposure on Growth, Physiological and biochemical Parameters of *Dracocephalum moldavica* L.

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Abstract

The increasing commercial production and the broad usage of engineered nanoparticles (ENPs) have led to concerns over the potential adverse impacts of these ENPs on biota in natural environments. Silver nanoparticles (AgNPs) are one of the most important and widely used ENPs which enter natural ecosystems. In this study we examined the effects of AgNPs on growth and some physiological parameters of *Dracocephalum moldavica* L. The experiment was conducted hydroponically in a AgNPs spiked solution. The AgNPs toxicity exhibited a decline in growth and chlorophyll content of *D. moldavica* at elevated concentrations (> 40 mg/L). AgNPs significantly induced oxidative stress by increasing H₂O₂ production in a concentration dependent manner. The phytotoxicity of AgNPs led to an increase in catalase and peroxidase activities and synthesis of antioxidant compounds such as carotenoids, proline and total soluble carbohydrate. The decline of chlorophyll content at highest AgNPs treatment (80 mg/L) was associated with disturbances in photosynthetic capacity which ultimately results in the reduction of *D. moldavica* growth. Increase carotenoids, proline and total soluble carbohydrate content suggest that compatible solutes may contribute to osmotic adjustment at the cellular level and enzyme protection stabilizing the structure of macromolecules and organelles. Our results indicate important new avenues of research for understanding the fate of AgNPs in hydroponic media, the interactions between AgNPs and *D. moldavica*.

Keywords: *Dracocephalum moldavica* L., Silver nanoparticles, Growth parameters, phytotoxicology

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Introduction

In nanotechnology, submicroscopic particles ranging from 1 to 100 nm in diameter are usually referred to as nanoparticles. Increasing production and use of nano-sized materials have

raised concerns about their possible impacts on environmental and human health (Colvin, 2003). However, our understanding of how ENPs may affect organisms within natural ecosystems lags far behind our rapidly increasing ability to engineer novel nanomaterials (Qian et al., 2013). Such nonmaterial on entering in ecosystem might have affected or effective growth parameters in plants.

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Nanoparticles interact with plants causing many morphological and physiological changes, depending on the properties of NPs. Efficacy of NPs is determined by their chemical composition, size, surface covering, reactivity, and most importantly the dose at which they are effective (Khodakovskaya et al. 2012). Therefore, researcher's findings suggested that the effects on nanoparticles on plants differ and they were mainly relying on the plant species and nanoparticles characteristics such as size, morphology, composition and physic-chemical properties (Ma et al. 2010). Some plants are capable of up taking and accumulating engineered nanomaterials. Efficacy of NPs depends on their concentration and varies from plants to plants. The effects of NPs on different plant species can be related to plant growth stages, method, and duration of exposure and depend on the ENS shape, size, chemical composition, concentration, surface structure, aggregation, and solubility. Finally, the interaction of plant cell with the NPs lead to the modification in associated plant growth pathways and modifications in gene modifications which these phenomena eventually affects plants growth and developments (Mustafa and Kumatsu, 2016).

During recent years' development and use of silver nanoparticles (AgNPs) has received enormous attention over other NPs due to their advantageous applications in biomedical, food industries, agriculture, textile industries, water treatment as an antimicrobial and antifungal agent. Therefore, such NPs can generate adverse biological effects in living organisms such as plants (Navaro et al., 2008).

There is an increasing amount of research on the toxicology of nanomaterials, however, how nanomaterials affect living organisms remains unknown (Navaro et al., 2008). Limited studies reported both positive and negative effects of NPs on higher plants. It was pointed out a correlation between the growth efficiency of crops and the concentration of the AgNPs once the smaller growth of the plants cultivated with AgNPs was observed which this growth inhibition can be due to the absorption of the AgNPs by the plants and the presence of dissolved Ag ions, which can also be toxic (Cristina et al., 2015). In another report, researchers have

evaluated the effects of the low AgNPs concentrations on rice growth and development. Results showed higher growth than the control plants, whereas at higher AgNPs concentrations, a minor growth was observed due to the alterations in the cell morphology and in the structural characteristics (Navaro et al., 2008).

The primary response of plants to nano metal stress like AgNPs can be lead to the generation of reactive oxygen species (ROS) which act as the major contributors to oxidative damage and associated toxicity (Vazquez et al., 2008; Yadav, 2010). These ROS are highly reactive and damage membrane lipids, proteins, pigments and nucleic acids, resulting in dramatic reductions of growth and productivity, and eventually causing the death of plants (Foyer et al. 1994; Sandalio et al., 2012). Plants living under oxidative stress of heavy metals and metallic nanoparticles exhibit adaptive biochemical responses such as production of antioxidant enzymes and compounds. Antioxidative defense systems fall into non-enzymatic (glutathione, soluble carbohydrates, proline and carotenoids) and also enzymatic defense, such as peroxidase (POD) and catalase (CAT), which protect plants against oxidative damage (Garg and Manchanda, 2009; Sandalio et al., 2012).

There are still many unresolved issues and challenges concerning the biological effects of nanoparticles especially in medicinal plants. Attentions to appropriate experimental design and interpretation are needed to provide a defensible scientific understanding of the biological effects of nanoparticles.

Dracocephalum moldavica L. which belong to the family Lamiaceae, is an annual, herbaceous, essential oil producing, spicy aromatic medicinal plant. It has been used in Iran for their culinary usefulness and medicinal properties (Haghighi pak et al., 2016). Therefore, extracts of the plants were screened for antioxidant properties in a battery of in vitro assays. The plant extracts demonstrated a wide range of antioxidant activities (Nazeruddin et al., 2012). Therefore, this study aimed to provide new information about phytotoxicology of AgNPs on growth parameters of *Dracocephalum moldavica* L. as higher plant and medicinal plant species.

Materials and Methods

Plant culture and AgNPs exposure

Seeds of *D. moldavica*, were obtained from the Pakan Bazr company, Isfahan, Iran. They were sterilized with 10% sodium hypochlorite solution (V/V) for 15 min and were rinsed at least 5 times with sterilized water. Seeds were germinated on distilled water moistened filter paper in Petri dishes for 4 d at room temperature, <math><20^{\circ}\text{C}</math>. After germination, seedlings were then transferred to 1-l polyethylene pots (four seedlings per pot) filled with culture media containing 45% cocopit, 30% perlite, and 25% sand. Plants were feeding with a 10 % Hoagland nutrient solution composed of 0.5 mM KNO_3 , 0.75 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM KH_2PO_4 , 0.2 mM MgSO_4 , 15 μM H_3BO_3 , 2 μM MnCl_2 , 1 μM ZnSO_4 , 0.5 μM CuSO_4 , 0.2 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 50 μM Fe-EDTA (pH 6.0). After cultivating the plants were amended for 10 days then the solutions with different AgNPs concentrations (0–80 mg/L) added for another 2 weeks. The AgNPs were purchased from Pishgaman Nanotechnology Company located in Mashhad, Iran. The particle diameter was approximately 10 nm according to the manufacturer's data and transmission electron microscopy (TEM) results. Upon addition to the medium, the AgNPs were dispersed by ultrasonic vibration. *D. moldavica* was grown in a greenhouse at (25 ± 5) $^{\circ}\text{C}$ under a sunlight intensity limited to $300 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 16 h of light/8 h of dark. Each treatment was replicated three times and each time, the pots were randomly arranged during the growth period.

Determination of photosynthetic pigments and plant biomass

After 3 weeks AgNPs exposure, the chlorophyll (Chl) contents (Chl *a*, Chl *b*, total Chl) and carotenoids were measured in 80% acetone extract of 0.2 g leaf tissue. Leaf was cut into small pieces, mixed thoroughly and grind with 25 ml of 80% cold acetone for 2 min. The homogenate was filtered through filter paper (Whatman No.1) and was made a volume of 25 ml with 80% cold acetone. The optical density of each solution was measured at 663, 645 and 470 nm against 80% acetone blank in 1.5 cm cell. The method of

Arnone (1949) was used to calculate the amount of photosynthetic pigments.

At harvest, plants were divided into root and shoot fractions. Root tissue samples were rinsed twice in deionized water to remove surface contaminants. Plants samples were air-dried in an oven at 70°C for 48 hours. Dried samples were weighted and ground in mortar to obtain homogeneous samples.

Estimation of Proline content

Proline colorimetric determination was preceded according to Bates et al. (1973) based on proline's reaction with ninhydrin. Dry leaves (100 mg) were homogenized in 10 ml of aqueous sulphosalicylic acid (3%). The homogenate was filtrated with Whatman filter paper. A two ml aliquot of the filtrate was mixed with an equal volume of acetic acid and ninhydrin and incubated for 1 h at 100°C . The reaction was terminated on ice bath and extracted with 4 ml of toluene. The extract was vortexed for 20 s and the chromophore containing toluene was aspirated from the aqueous phase and absorbance determined photometrically at 520 nm (Tomas 302, USA) using toluene for a blank.

Extraction and determination of total soluble carbohydrate

For determination of total soluble carbohydrate content, 50 mg of dry shoot powder was extracted using 10 ml of ethanol: distilled water (8:2; v/v), and supernatant was collected after twice centrifugation at 1480 *g*. Total soluble sugar content was estimated calorimetrically using phenol sulfuric acid method described by Dubois et al. (1956).

Measurement of hydrogen peroxide (H_2O_2)

H_2O_2 content was determined as described by Velikova et al (2000). Fresh shoots (0.5 g) were homogenized in ice bath with 5 ml 0.1% (w/v) trichloroacetic (TCA). The homogenate was centrifuged at $12,000 \times g$ for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. H_2O_2 concentration was estimated based on the absorbance of the supernatant at 390 nm. All colorimetric measurements (including enzyme

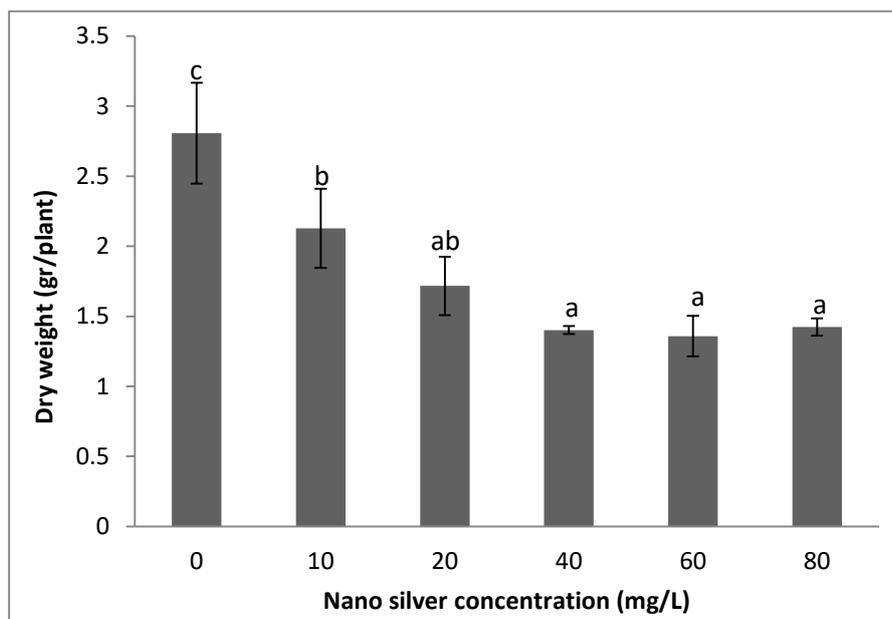


Fig. 1. *Dracocephalum moldavica* biomass after 3 weeks grown on hydroponic solution with different engineered silver nanoparticle concentration. Values represent mean \pm SD. Different letters indicate significant differences at the 5 % level according to the Duncan test.

activities) were made at 20°C in a Bausch & Lomb 70 spectrophotometer.

Peroxidase assay

Peroxidase (POD, EC 1.11.1.7) activity was measured according to Chance and Maehly (1995). The reaction mixture (3.0 ml final volume) consisted of 50 μ l of 10 mM guaiacol, 2.9 ml of 50 mM K-phosphate buffer, pH 7.0, 10 μ l of 40 mM H₂O₂. A 40 μ l aliquot of the crude enzyme extract was then added to start the reaction. The activity of the mixture was determined spectrophotometrically at 470 nm after 1 min at 20°C. Enzyme activity was calculated by using the extinction coefficient of (26.6 mM⁻¹ Cm⁻¹).

Catalase assay

Catalase activity was assayed by following the decline in absorbance of H₂O₂ at 240 nm according to the method of Aebi (1984). The reaction mixture consisted of 100 μ l of enzyme extract in 50 mM sodium phosphate buffer (pH 7.0). The reaction was started by addition of 20 mM H₂O₂, and its consumption was measured for 2 min.

Statistical analysis

Statistical analysis was performed using the version 16 of SPSS statistical package. One-way analysis of variance (ANOVA) was performed to test the significant differences ($P < 0.05$) between the treatment and control means. Duncan's multiple range test (DMRT) was also performed to compare among the groups for significant differences. All the values presented in this paper were the means of three replicates. \pm standard error (S.E).

Results

Plant Growth

Generally, the biomass of plants may give an important index for their identifying them as a tolerant or sensitive to subjected stress. Therefore, this parameter should decrease significantly at the threshold concentration of inhibiting plant growth. In this study, the total dry biomass was measured at the end of the experiment, and presented in Fig. 1. During the 21-day exposure to AgNPs, the toxicity symptoms were observed in *D. moldavica* shoots at higher treatments (> 40 mg/L). It is revealed that the dry weight of plants was significantly ($p < 0.05$) decreased at AgNPs supply levels of 10–80 mg/L

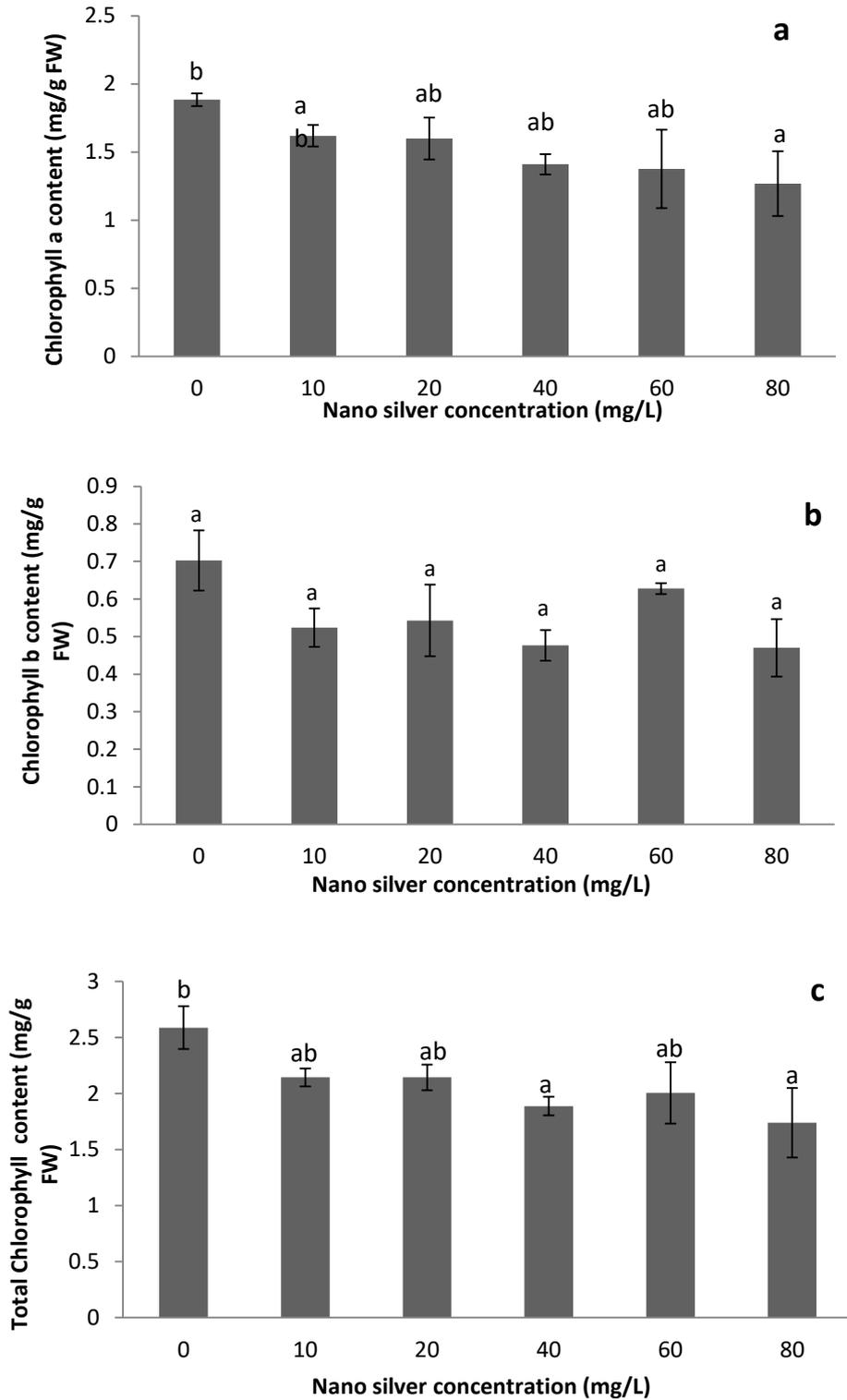


Fig. II. Chlorophyll a, chlorophyll b and total chlorophyll contents in *Dracocephalum moldavica* treated with different engineered silver nanoparticle concentration. Values represent mean \pm SD. Different letters indicate significant differences at the 5 % level according to the Duncan test.

compared to the control. The maximum plant dry weight dramatically decreased at AgNPs levels higher than 40 mg/L (Fig. I). The maximum plant

dry weight at 80 mg AgNPs /L were only about 50 % of the control studied plant.

Plant Photosynthesis

Chlorophyll and Carotenoid Content

The contents of Chl-a, Chl-b and total Chl all displayed decreasing trend with the increase of AgNPs concentration (0–80 mg/L) (Fig. II a-c). The mean Chl-a contents in the leaves of the *D. moldavica* was 1.88, 1.61, 1.59, 1.41, 1.37 and 1.26 mg/g FW in control, 20, 40, 60 and 80 mg/L of AgNPs treatments, respectively while Chl-b contents were 0.692, 0.702, 0.523, 0.542, 0.476, 0.627 and 0.474 mg g⁻¹, respectively. In highest level of AgNPs treatment (80 mg/L), Chl-a, Chl-b and total Chl declined by 32.65%, 33.10% and 32.77%, respectively comparing with control. A positive correlation was found between carotenoids and AgNPs applications in *D. moldavica* shoots. The maximum induction was observed in treatments contain 80 mg/L AgNPs, which it showed 3.69 mg/g (FW) (Fig III).

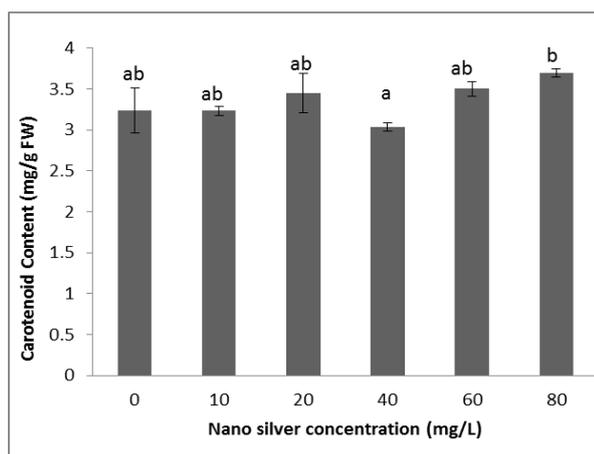


Fig. III. Effect of different engineered silver nanoparticle concentration in the medium on carotenoids content in leaves of *Dracocephalum moldavica*. Values represent mean \pm SD. Different letters indicate significant differences at the 5% level according to the Duncan test.

Free proline accumulation

The results pertaining to the effect of AgNPs on proline content is presented in figure 4. The 10 to 60 mg/L of AgNPs did not significantly effect on the proline content of *D. moldavica*. The greatest increase in proline content was occurred in medium contain 60 mg/L of AgNPs (Fig. IV). The proline accumulation at 80 mg/L AgNPs increased proline content to 5.8 fold of the control.

Soluble carbohydrate content

The effect of silver nanoparticles on soluble carbohydrate content of the *D. moldavica* was illustrated on Fig. V. The carbohydrate content of *D. moldavica* was found to be in accordance with the photosynthetic pigments for corresponding AgNPs treatment. For 80 mg/L AgNPs treatment, *D. moldavica* showed 3.7 fold increases in soluble carbohydrate over control.

Lipid peroxidation

H₂O₂ activity induced by AgNPs stress in the shoots increased in a concentration dependent manner and was more in roots than in shoots. It can be suggesting that AgNPs cause reduction of *D. moldavica* growth by inducing oxidative stress and increased generation of H₂O₂ related to enhanced lipid peroxidation.

Antioxidative enzymes

In the present study, activity of antioxidant enzymes profoundly indicated that there was a clear relation between the mechanism of tolerance and susceptibility of *D. moldavica* under as stress. So, comparing the activities of these two enzymatic antioxidants, it is clearly evident that the uptake of AgNPs induced a strong antioxidative response in *D. moldavica* (Fig VI and VII)

Discussion

Nanoparticles interact with plants causing many morphological and physiological changes, depending on the properties of NPs. Efficiency of NPs is determined by their chemical composition, size, surface covering, reactivity, and most importantly the dose at which they are effective (Khodakovskaya et al. 2012). Researchers from their findings suggested both positive and negative effects on plant growth and development and the impact of engineered nanoparticles (ENPs) on plants depends on the composition, concentration, size, and physical and chemical properties of ENPs as well as plant species (Ma et al. 2010).

Reduction of biomass of root and shoot is a typical of response to toxic metals, metalloids and metallic nanoparticles (Mustafa and Kumatsu, 2016). Reduced root and shoot growth in response

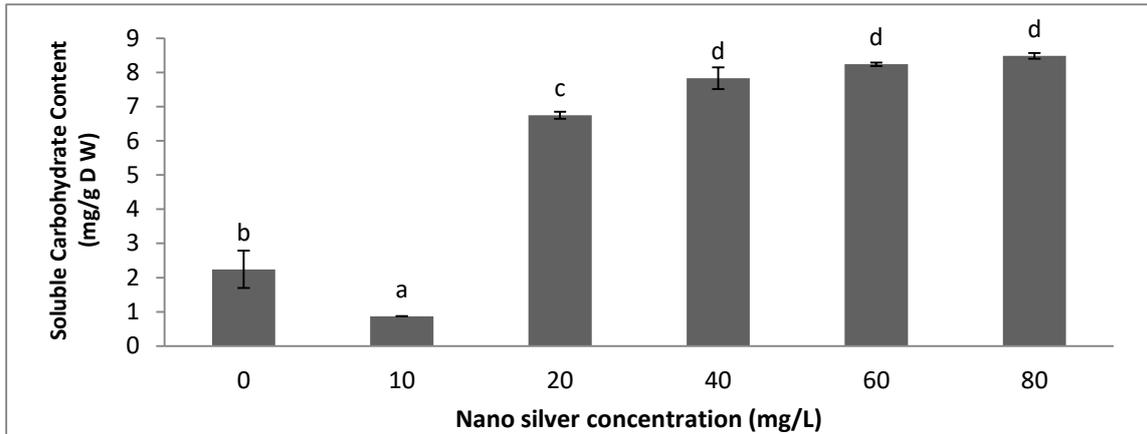


Fig. IV. *Dracocephalum moldavica* soluble carbohydrate content after 3 weeks grown on hydroponic solution with different engineered silver nanoparticle concentration. Values represent mean \pm SD. Different letters indicate significant differences at the 5 % level according to the Duncan test.

to AgNPs exposure has been reported by a number of investigators in other plants (Qian et al., 2013; Cristina et al., 2015). In this study, the reduction of *D. moldavica* biomass due to AgNPs exposure was occurred although it was noted that at the highest AgNPs treatment of 80 mg/L it still has >50% plant biomass. Based on this growth trait, it is suggested that *D. moldavica* can tolerate against high AgNPs treatments (Fig. 1). This tolerance to AgNPs could be related to some physiological and biochemical adaptation strategies, which have been described to be heritable, often as a result of multiple or single gene differences which lead to different antioxidative responses by plants like *D. moldavica*. The same finding has also been observed in Eleven Wetland and some other terrestrial plants (Qian et al., 2013).

Plant Photosynthesis

Plant growth is inhibited as a consequence of reduction in leaf photosynthetic rate under different environmental stress (Rodriguez-Serrano et al., 2009). The inhibited photosynthesis involves decreasing the chlorophyll content and damaging leaf PSII (Mac Farlane, 2003) which is directly attributed to stress tolerant ability of plants. Therefore, the content of photosynthetic pigments can determine the capacity of photosynthesis in AgNPs stressed plants and it gives an important index to identify tolerant plants. In this study, the chlorophyll content in *D. moldavica*, did not show statistical differences,

especially when plants were exposed to 40 to 80 mg/L AgNPs (Fig. 2a-c).

Our results of decrease in chlorophyll content at highest AgNPs treatment corroborated with the findings of Ma et al. (2010) who also found a decrease in chlorophyll content with affecting nanoCeO₂ to *Arabidopsis*. The loss in chlorophyll content can consequently lead to disruption of photosynthetic machinery. Chlorosis symptoms appeared at the highest AgNPs supply, which could be a direct result of the increased stress concentrations action on membranes.

Carotenoids act as light-harvesting pigments as well, and can protect chlorophyll and membrane destruction by quenching triplet chlorophyll and removing oxygen from the excited chlorophyll-oxygen complex (Krinsky, 1994). In this study, carotenoid content of *D. moldavica* increased with increasing AgNPs concentrations which lead to greater protection of this plant against reactive oxygen species (ROS). Therefore, carotenoids protective function can be partly due to antioxidant properties against free radicals produced by metals or metallic nanoparticles (Sakihama and Yamasaki, 2002).

Free proline accumulation

Accumulation of proline as compatible solutes is another strategy that plant adopt to withstand stress conditions (Parida & Das, 2005). Proline, an imino acid is well known to get accumulated in wide variety of organisms ranging

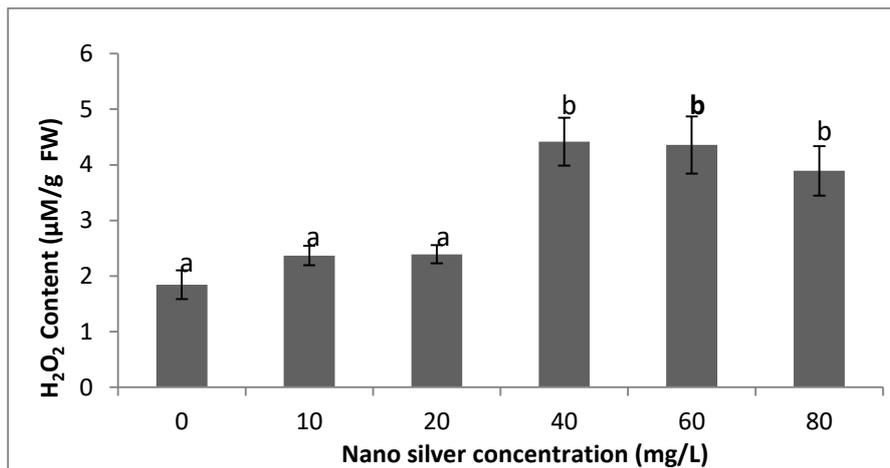


Fig. V. Effect of different engineered silver nanoparticle concentration in the medium on H₂O₂ content in leaves of *Dracocephalum moldavica*. Values represent mean ± SD. Different letters indicate significant differences at the 5 % level according to the Duncan test.

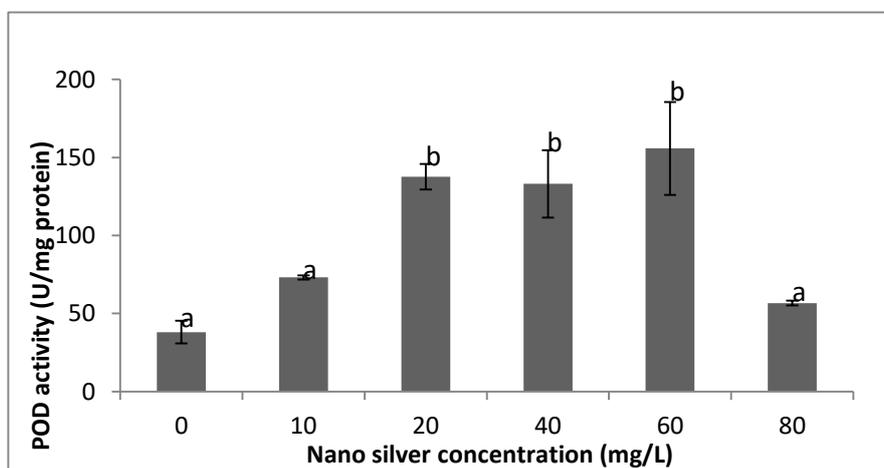


Fig VI. Effect of different engineered silver nanoparticle concentration in the medium on peroxidase (POD) activity in shoots of *Dracocephalum moldavica*. Values represent mean ± SD. Different letters indicate significant differences at the 5 % level according to the Duncan test.

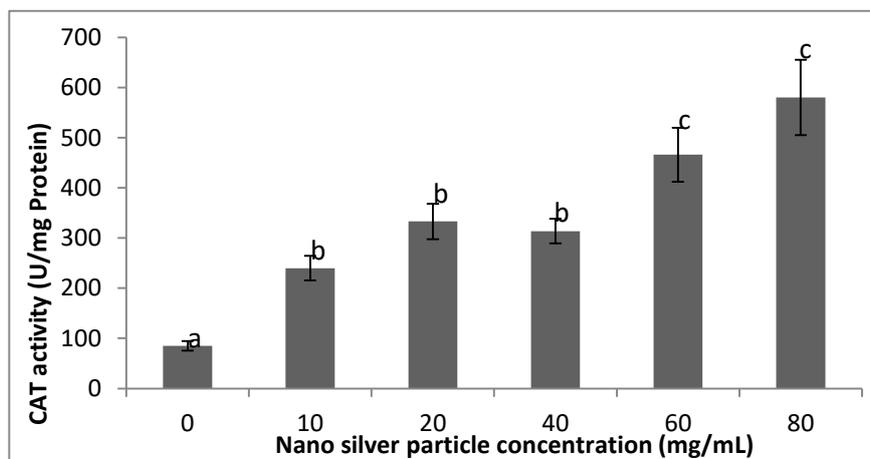


Fig. VII. Effect of different engineered silver nanoparticle concentration in the medium on catalase (CAT) activity in shoots of *Dracocephalum moldavica*. Values represent mean ± SD. Different letters indicate significant differences at the 5 % level according to the Duncan test.

abiotic stress (Saradhi et al., 1993). In *D. moldavica*, proline concentration increased considerably under AgNPs stress, which indicates that the overproduction of this compound is a non-specific response. Increase in proline content may be either due to *de novo* synthesis or decreased degradation or both (Kasai et al., 1998). It has been often suggested that proline accumulation may contribute to osmotic adjustment at the cellular level and enzyme protection stabilizing the structure of macromolecules and organelles. Then it can serve as an organic nitrogen reserve ready to be used after stress relief to sustain both amino acid and protein synthesis. Proline accumulation in shoots of Basil (*Ocimum basilicum*) in response to silica nanoparticles toxicity has been demonstrated by Kalteh et al., (2014). Similar results of increasing proline content by SiO₂ nanoparticle was also reported by Zarafshar (2015) in wild Pear.

Soluble carbohydrate content

It is believed that under heavy metal stress accumulation of sugar along with other compatible solutes contribute to an osmotic adjustment allow the plants to minimize sufficient storage reserves to support basal metabolism under stressed environment (Smeekens, 2000). The major functions of sugars are osmoprotection, osmotic adjustment, carbon storage, and radical scavenging (Parida & Das, 2005). Abiotic stress like salinity and heavy metals increased reducing sugars and sucrose in plants (Dubey & Singh, 1999). In this study, the concentrations of soluble sugars increased in *D. moldavica* shoots with increasing levels of AgNPs in the medium (Fig. 5). The increase in sugar concentration may be a result from the degradation of starch. Starch may play an important role in accumulation of soluble sugars in cells. A similar result of increasing proline content by AgNPs was also reported by Salama (2012) in common bean (*phaseolus vulgaris* L.) and corn (*Zea mays* L.).

Lipid peroxidation

Impacts of AgNPs concentrations were evaluated to assess AgNPs induced oxidative damage by measuring changes in the hydrogen peroxide (H₂O₂) contents. H₂O₂ is a product of superoxide dismutase reaction, with greater levels

indicating greater oxidative stress. Its content in plants exposed to adverse environmental conditions is a reliable indicator of free radical formation in the tissues, and it is currently used as an index of oxidative damages in biological systems.

Antioxidative enzymes

Cells are normally protected against ROS by the operation of intricate antioxidant systems, comprising enzymatic systems such as POD and CAT. The accumulation of H₂O₂ during oxidative stress is prevented by some antioxidative enzymes like POD and CAT by reduction to H₂O. Furthermore, POD participates in lignin biosynthesis which it could build up a physiological barrier against toxic heavy metals (Radotic et al., 2000; Hegedus et al., 2001). In this research, POD and CAT activities, compared with control, was significantly higher in *D. moldavica*. Therefore, a remarkable increase in the activities of POD and CAT at the high AgNPs levels are usually regarded as an indicator of tolerance in the *D. moldavica*. So, these results suggest that AgNPs induced increases in the levels of antioxidant enzymes may represent a key defensive mechanism against oxidative stress. Meanwhile, healthily grew of *D. moldavica* was associated with antioxidant defenses which maintaining plant growth

Conclusion

The present study demonstrated the effect of silver nanoparticles on medicinal plant species *D. moldavica*. The presence of AgNPs affects growth of *D. moldavica* at different concentrations. The maximum effect was found at 80 mg/L for this medicinal plant. Beyond this concentration the growth was inhibited. The effective growth at certain optimum concentration and inhibited growth beyond this concentration may be attributed to the accumulation and uptake of AgNPs and H₂O₂ by the roots. It was found that the accumulation and uptake of nanoparticles was dependent on the exposure concentration. It is possible that AgNPs resistance of *D. moldavica* was associated with its ability to maintain a coordinated increase in the concentration of carotenoid, proline and soluble carbohydrates and enhancement of antioxidative

enzymes such as POD and CAT resulting in higher plant stability

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