Effect of biologically synthesized silver nanoparticles on *Melissa officinalis* L.: Evaluation of growth parameters, secondary metabolites, and antioxidant enzymes

Asiyeh Shavalibor and Sedigheh Esmaeilzadeh Bahabadi*

Department of Biology, Faculty of Basic Sciences, University of Zabol, Zabol, Iran

Abstract

Using bionanoparticles plays an important role in increasing agricultural productivity. In recent years, the use of nanoparticles in plants has been considered as pesticides, protective agents, and nutrients. The present study investigated the effects of different concentrations of bio-synthesized silver nanoparticles (Ag NPs) on growth indices, secondary metabolites, proline, carbohydrates, and antioxidant enzymes activity of *Melissa Officnalis* L. The plantlets were treated with different concentrations of Ag NPs (0, 20, 60, and 100 ppm) at eight-leaf stage**.** Seedlings performances in terms of growth, antioxidant defense, and secondary metabolites content were studied under three different concentrations (20, 60, and 100 ppm) on different dates of Ag NPs application showed growth promotion effects on *M. officinalis.* The maximum growth rate and photosynthesis pigments contents were observed at 60 ppm Ag NPs concentration on day 15. Proline and carbohydrate contents increased significantly compared with the control by all concentrations of Ag NPs which exhibited time-dependent response. The Ag NPs also enhanced secondary metabolites content in *M. officinalis* seedlings. The highest amount of rosmarinic acid (about 50 mg/g DW) was obtained from those plants treated with 60 and 100 ppm of Ag NPs on day 15 which was about 3-fold higher than control. Up-regulation of antioxidant enzymes was observed with Ag NPs which led to a decrease in MDA content. Our findings confirmed for the first time that biologically synthesized Ag NPs at specific levels has significant growth promotion effects as well as increased production of valuable secondary metabolites.

Keywords: antioxidant enzymes*, Melissa officinalis*, rosmarinic acid, silver nanoparticles**,** secondary metabolites

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Introduction

Nanotechnology plays an important role in improving agricultural productivity. Silver nanoparticles (Ag NPs) have been widely used in different fields such as dentistry, clothing, photography, catalysis, electronics, and pharmaceutical and food industry (Nowack et al., 2011). At the present, several chemical and physical routes and recently green synthesis procedure are known for preparation of Ag NPs (Sathishkumar et al., 2016). However, the biological method represents a non-toxic and

* *Corresponding Author*
E-mail Address: esmaeilzadeh@uoz.ac.ir
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environmentally acceptable route (Gurunathan et al., 2009). The use of plants and their extracts as reducing agents for the production assembly of Ag NPs is very feasible, cost-effective, and eco-friendly.

Nanomaterials have been shown to have both positive and negative effects on plant growth and metabolism (Ma et al., 2010). The positive effects of Ag NPs such as growth promotion and biochemical attributes (chlorophyll, carbohydrate, protein contents, and antioxidant enzymes) have been reported on Indian mustard, common bean, and maize (Salama et al., 2012; Gruyer et al., 2013). However, negative effects of Ag NPs have also been reported on plants. For instance, it has been reported that Ag NPs treatments decreased growth in *Triticum aestivum* and *Oryza sativa* (Vannini et al., 2014; Nair and Chung, 2014). Altogether, these findings suggest that the effect of Ag NPs on plants strongly depends on concentration, form of nanoparticles, exposure time, and plant species. It has been reported that nanoparticles can relieve reactive oxygen species (ROS) accumulation and malondialdehyde (MDA) content by induction of antioxidant enzyme activities (Lei et al., 2008). Also, phenols and flavonoids are reported to be induced by nanoparticles (Ghorbanpour, 2015; Homaee and Ehsanpou, 2015).

Nanoparticles have been shown to be effective in inducing secondary metabolites and physiological responses (Ghorbanpour and Hatami, 2015). To date, there are few reports concerning the application of nanomaterials as elicitors to induce the production of secondary metabolites. Recently, Ghorbanpour and Hadian (2015) reported that carbon nanotubes can act as a novel elicitor for production of rosmarinic acid (RA) and caffeic acid in *Satureja khuzestanica*.

Lemon balm (*Melissa officinalis* L.) is an aromatic perennial herb that belongs to the family Lamiaceae. It has therapeutic properties, such as sedative, carminative, antispasmodic, antibacterial, antioxidant, antiviral, and neuroprotective effects (Kamdem et al., 2013; Pereira et al., 2015). To date, there have been only a few studies reporting on the effect of biologically synthesized Ag NPs on medicinal plants. Hence, in the present study, we investigated the potential effect of different concentrations of biosynthesized Ag NPs on the growth and some physiological parameters of *M. officinalis* at different times. To understand the possible toxic effects of Ag NPs, MDA content, antioxidant enzymes activities, phenolics, flavonoids, anthocyanins, RA, carotenoids, carbohydrate, and proline were evaluated.

Materials and Methods

Plant materials and growth conditions

Seeds of *M. officinalis* were planted in plots filled with perlite and sand (1:3) and kept in the greenhouse under 28 ℃, 16/8 h light/dark photoperiod and 75% relative humidity.

The plants were sprayed with different concentrations of Ag NPs (20, 60, and 100 ppm). All measurements were performed after 5, 10, 15, and 20 days of treatments.

Ag NPs biosynthesis and characterizations

Ag NPs were synthesized from $AgNO_3$ using *Prosopis farcta* fruit extract. The biologically synthesized Ag NPs were characterized by UV visible spectroscopy. Furthermore, they were examined by UV-visible spectroscopy measurements followed by X-ray diffraction spectroscopy (XRD) and transmission electron microscopy (TEM) (Salari et al., 2019).

Determination of photosynthetic pigment contents

Content of chlorophyll and carotenoids were determined according to the methods of Cock et al. (1976). Briefly, the leaves were cut in small pieces and immersed in 80% acetone solvent to extract pigments. The extraction solvent was diluted and measured with a spectrophotometer at 663, 645, and 440.5 nm. The contents of pigments were calculated as follows:

$C_a = 0.0127 \text{ X } D663 - 0.00269 \cdot D645$

C_b (mg g^{-1} fresh leaf) $= 0.0299 \cdot D645 - 0.00468 \cdot D663$

C_{car} (mg g^{-1} fresh leaf) = 0.004695 · D440.5 − 0.000268 (Ca + Cb).

where C_a, C_b are contents of chlorophyll a and b (mg g^{-1} fresh leaf) and Ccar is the content of carotenoid (mg g^{-1} fresh leaf).

Determination of soluble carbohydrate and protein content

Total soluble sugar content was determined using anthrone reagent and glucose as standard (Roe, 1955). Briefly, 0.1 g dry weight of leaf tissue powder was ground and extracted with 2.5 ml of 80% (v/v) ethanol at 90 ℃ for 60 min, followed by centrifugation at 10,000 g at 4 ℃ for 10 minutes. The process was repeated for complete extraction. Soluble carbohydrate contents were expressed as mg g^{-1} DW. Protein content was estimated spectrophotometrically by Bradford method (Bradford, 1976). Briefly, samples (0.5 g) were homogenized in 2.5 ml of 50 mM phosphate buffer (pH 7) containing 1 M ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% polyvinyl pyrrolidone (PVP). The homogenate solution was centrifuged at 20,000 g at 4 ℃ for 20 min and the supernatant was used for measurement of total soluble protein, expressed as mg g^{-1} DW.

Determination of proline content

Proline content of leaves was determined according to Bates et al. (1973) and 0.1 g of leaves was homogenized in 10 mL of aqueous 3% sulfosalicylic acid and the homogenate was filtered. The filtrate (2 mL) was mixed with 2 mL fresh acid ninhydrin solution and 2 mL of glacial acetic acid and then with 4 mL toluene. The absorbance of the colored solutions was measured at 520 nm.

Quantification of total phenol, flavonoid, anthocyanin, and RA content

Total phenol content was determined spectrophotometrically, according to the Folin-Ciocalteu method, using Gallic acid (0–1000 µM) as the standard and expressed as Gallic acid equivalents (GAE) per gram fresh weight (Singleton et al., 1999). Flavonoid content of each extract was determined following colorimetric method. Briefly, 20 µL of each extract were separately mixed with 20 µL of 10% aluminium chloride, 20 µL of 1 M potassium acetate, and 180 µL of distilled water, and left at room temperature for 30 min. The absorbance of the reaction was recorded at 415 nm. The calibration curve was prepared using Rutin methanolic solutions at concentrations of 12.5 to 100 µg mL^{-1}. Flavonoid content was expressed as mg Rutin equivalents per gram fresh weight (Cheng et al., 2002).

Anthocyanin concentration was determined by the method of Wagner (1979). Briefly, 0.1 g of fresh leaf was homogenized in 10 ml acidified methanol and kept at 25 ℃ for 24 h in the dark conditions. The homogenate was centrifuged at 4000 rpm for 10 min and the absorbance of each supernatant was determined at 550 nm. Total anthocyanin content was calculated using an extinction coefficient of 33,000 ($mM^{-1}cm^{-1}$).

RA extraction was performed in accordance with Wang et al. (2004) method with a few modifications. In brief, 100 mg dry tissue was ground and suspended in 10 mL of 30% ethanol, and was sonicated for 15 minutes. Then, the solution was centrifuged for 20 minutes at 4,000 rpm and 25 ℃. All samples were filtered through a 0.45 µm filter. The presence of RA in the samples was verified by HPLC with commercially available standard.

Determination of lipid peroxidation

The level of lipid peroxidation was measured in terms of MDA concentration that was determined by the method of Correia et al. (2006). Mature fresh leaves (0.15 g) were homogenized with 2 ml of ice-cold 50 mM phosphate buffer (pH 7.8) and 5 ml of 0.5% thiobarbituric acid (TBA). The mixture was heated in a water bath shaker at 100 ℃ for 10 min, and quickly cooled in an ice bath. The samples were centrifuged at 3,000 g for 15 min. The absorbance was measured at 532 nm.

Determination of antioxidant enzymes activity

SOD and CAT were assayed by the method described by Sankar et al. (2007) with a few modifications. For determination of SOD activity,

leaves (0.1 g) were homogenized in 3 mL of phosphate buffer (50 mM, pH 7.8) containing 1% polyvinylpolypyrrolidone. The homogenate was then centrifuged for 15 min at 10,000 g. Reaction mixture consisted of extraction buffer (0.1 mM EDTA, 50 mM Na_2CO_3, pH 10.2), 75 mM nitroblue tetrazolium chloride (NBT), 12 mM L-methionine, 4 mM riboflavin, and 0.2 mL of the enzyme extract. The amount of enzyme that results in 50% inhibition of the rate of NBT reduction at 560 nm was defined as one unit of SOD activity and expressed against mg protein of the extract.

For measurement of CAT activity, leaves (0.1 g) were extracted in 3 mL of sodium phosphate buffer (25 mM, pH 6.8) and centrifuged (12,000 rpm) for 20 min. The reaction mixture contained 1.0 ml of 50 mM Tris–Hydrochloric acid buffer (pH 7.0), 0.2 ml of 200 mM H_2O_2, 0.1 ml of enzyme extract, and 1.7 ml deionized water. Activity of CAT was monitored by measuring decrease in the absorbance at 240 nm due to H_2O_2 consumption, against mg protein.

POX activity was determined according to the method of Abeles and Biles (1991) with a few modifications. The reaction mixture contained 50 mM phosphate buffer (pH 6.8), 15 mM guaiacol and, 5 mM H_2O_2. Addition of H_2O_2 initiated the reaction and the increase in the absorbance at 470 nm was determined for 1 min. The extinction coefficient of 26.6 mM^{-1} $cm^{-1}$was used to calculate the amount of POX activity.

Statistical Analysis

The results were presented as means ± standard error. A one-way analysis of variance (ANOVA) was performed using SAS software. The significance of the difference between treatment means was determined by Duncan's Multiple Range Test at $p< 0.05$.

Results

Biological synthesis of Ag NPs and their characterization

Ag NPs were biologically synthesized using the *Prosopis farcta* fruit extract. Furthermore, the biologically synthesized Ag NPs were examined by

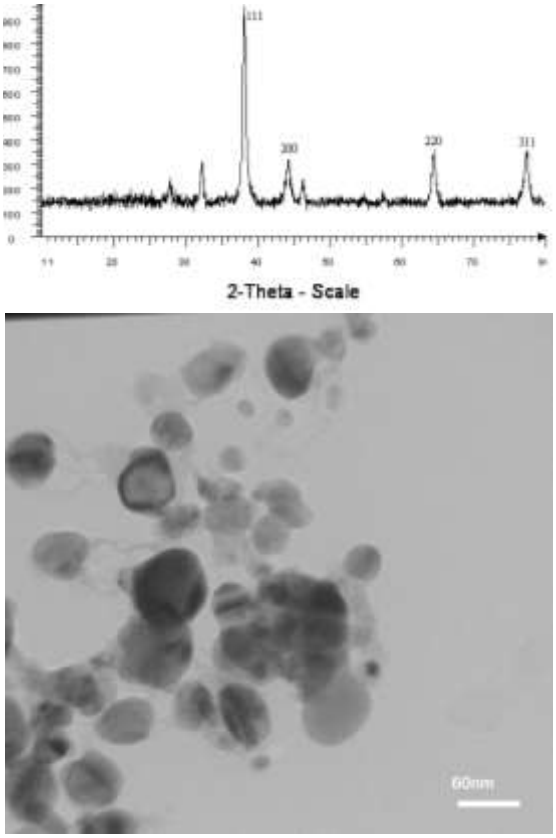

Fig. I. XRD pattern and TEM image biosynthesized Ag NPs

UV-visible spectroscopy measurements followed by XRD and TEM (Fig. I). The influence of process variables such as temperature, time of evolution, and extract concentration was also investigated to optimize the biosynthesis of silver nanoparticles. The average size of synthesized silver nanoparticles was 12.68 nm (10.26-14.65 nm). After the addition of $AgNO_3$ to the extract and incubation for 6 h, the extract color was turned dark brown showing the formation of Ag NPs in the reaction solution probably as a result of the excitation of surface plasmon resonance (SPR) bands (Mulvaney, 1996).

Effect of Ag NPs on seedling growth

Ag NPs positively influenced the growth and development of seedlings (Fig. II). Compared with controls, significant gradual increases in shoot weight were observed with increasing Ag NPs concentration over time. After 20 days of treatment, root weight increased compared with the control. Maximum increase in shoot and root growth was observed under 60 ppm Ag NPs treatment on day 15. Shoot and root length were also significantly affected by exposure to Ag NPs.

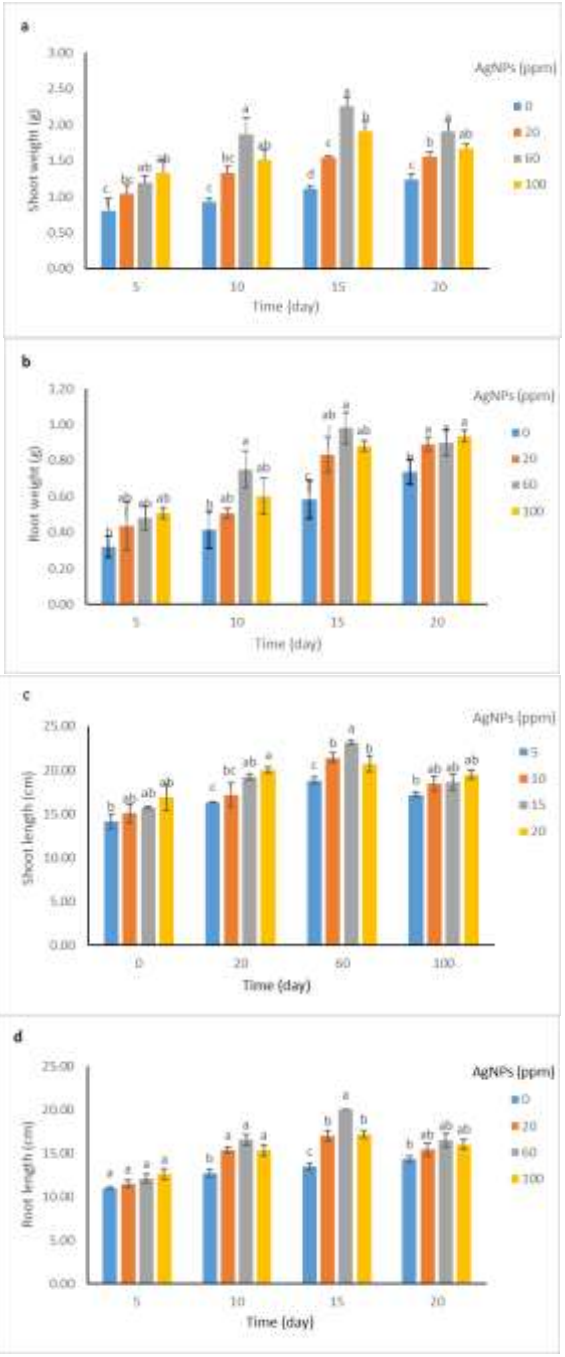

Fig. II. Effect of Ag NPs on seedling shoot weight (a), root weight (b), shoot length (c), and root length (d) of *M. officinalis*; different letter(s) indicate significant differences between the control and treatments at the same time ($p<0.05$).

After 10-day exposure, shoot and root of *M. officinalis* significantly increased in the treatments compared with the controls although there was no significant increase after 20-day exposure.

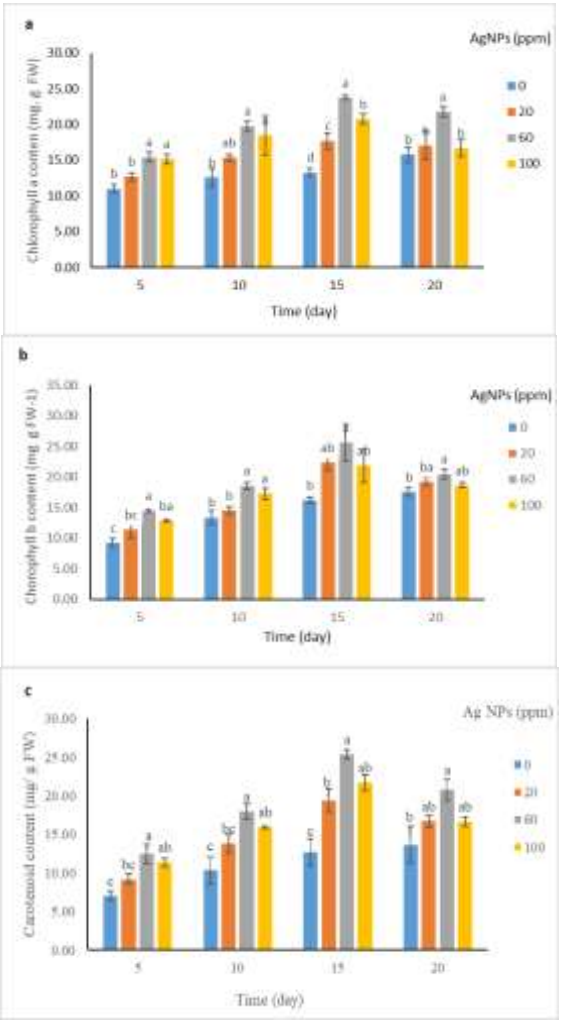

Fig. III. Effect of Ag NPs on chlorophyll a (a), chlorophyll b (b), and carotenoid (c) contents of *M. officinalis*; different letter(s) indicate significant differences between the control and treatments at the same time ($p<0.05$).

Effects of Ag NPs on photosynthetic pigments

Changes in the photosynthetic pigment contents were induced by different Ag NPs concentrations. As shown in Fig. (III) chlorophyll a, b, and carotenoid contents of *M. officinalis* were not significantly affected by 20 ppm Ag NPs at 5 and 10-day exposure compard with the control. Photosynthetic pigments in 40, 60, and 100 ppm Ag NPs treatments were significantly higher than those of the control at 5, 10, and 15-day exposure. Maximum contents of photosynthetic pigments were observed in plants treated with 60 ppm Ag NPs on day 15.

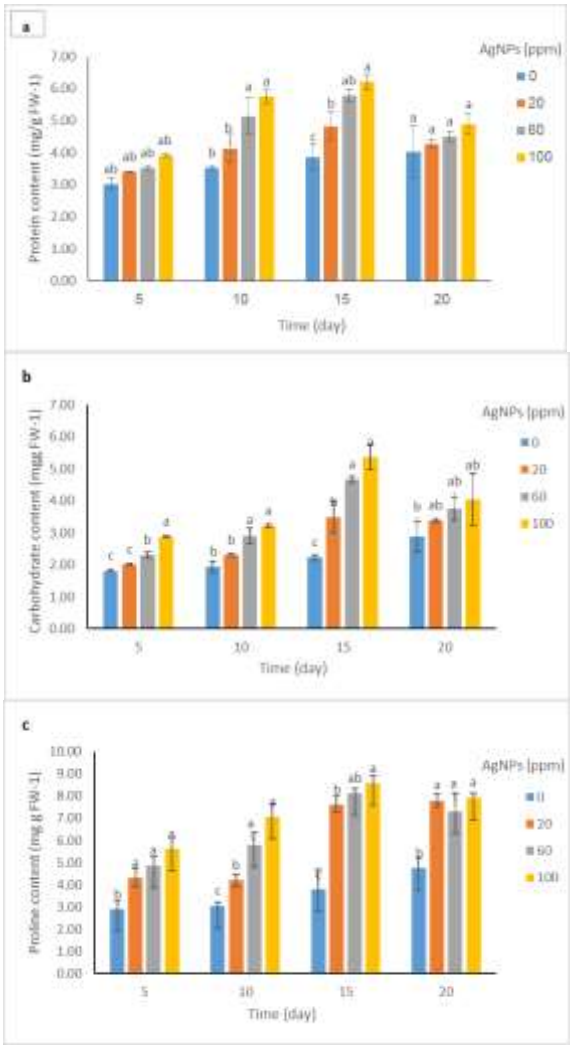

Fig. IV. Effect of Ag NPs on protein (a), carbohydrate (b), and proline (c) contents of *M. officinalis*; different letter(s) indicate significant differences between the control and treatments at the same time ($p<0.05$).

Effects of Ag NPs on protein, carbohydrate, and proline contents

The effect of Ag NPs treatments on *M. officinalis* protein and carbohydrate contents are depicted in Fig. (IV). As shown in the figure, exposure to different concentrations of Ag NPs at fifth day did not result in any significant change in total protein and carbohydrate contents of *M. officinalis* plants as compared with the control. However, total protein and carbohydrate contents increased significantly upon exposure to 40, 60 and 100 ppm of Ag NPs on day 10. Protein and carbohydrate contents increased with increasing the concentrations of Ag NPs on day 15 although no significant changes compared with the control

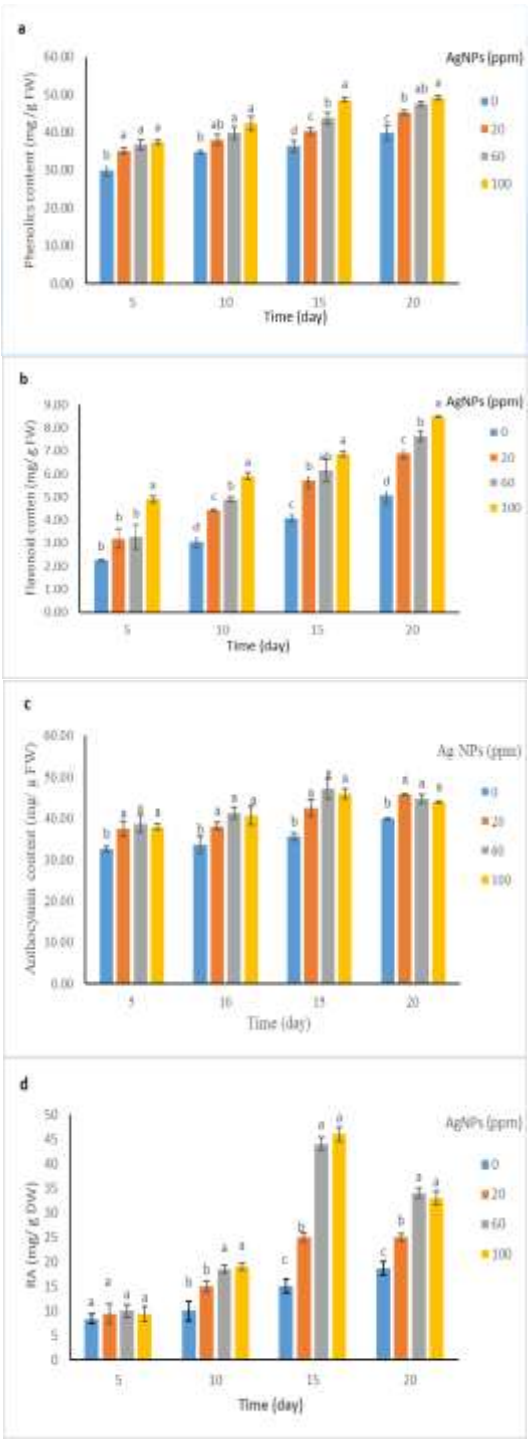

Fig. V. Effect of Ag NPs on phenolics (a), flavonoids (b), anthocyanin (c), and rosmarinic acid (d) contents of *M. officinalis*; different letter(s) indicate significant differences between the control and treatments at the same time ($p<0.05$).

plants were observed on day 20. The highest amounts of protein and carbohydrate were observed in plants treated with 60 and 100 ppm Ag NPs on day 15.

Effects of Ag NPs on secondary metabolites

Ag NPs treatments resulted in a significant increase in total phenol contents at all concentrations and exposure times (Fig. V. a). Similarly, flavonoids content also increased in a concentration- and time-dependent manner (Fig. V. b). The highest total flavonoids content was observed in plants exposed to 100 ppm Ag NPs on day 20.

Anthocyanin content in plants treated with Ag NPs significantly increased at all concentrations and times, hitting a peak at 60 ppm Ag NPs on day 15 (Fig. V. c).

The detection of RA was performed using HPLC analysis. As shown in Fig. (V. d), over 10-day exposure to Ag NPs, RA production in *M. officinalis* plants increased significantly with increasing Ag NPs levels. The highest amount of RA was obtained in the plants treated with 60 and 100 ppm of Ag NPs on day 15 which was about 3-fold higher than control.

Effects of Ag NPs on MDA content and antioxidant enzyme activities

As shown in Fig. (VI), MDA content slightly increased with increasing Ag NPs concentrations, over 5 and 10 days of exposure. In contrast, when plants were exposed to 15 days, MDA content decreased. There was no significant influence on MDA on day 20**.** Under stress, plants produce excess of ROS. Plants have several mechanisms of defense against stress. Scavenging or detoxification of excess ROS is achieved by an efficient antioxidant system including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). The antioxidant enzyme activities are depicted in Fig. (VI).

In this study, Ag NPs at all concentrations, upregulated the activity of SOD, CAT, and POX which exhibited time-dependent response. The increase observed in the enzyme activities could be one of the major protection mechanisms against increase in ROS generation. Based on our results, the increased SOD activities were coupled with increases in POD and CAT activities.

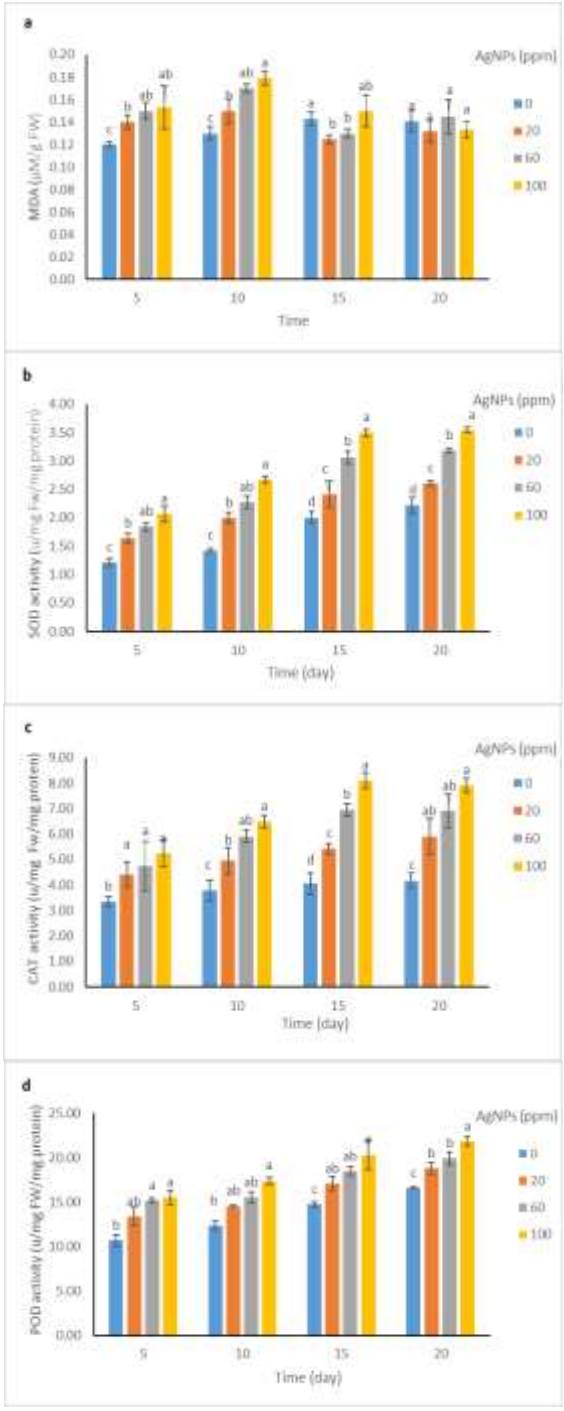

Fig. VI. MDA concentrations (a), activities of SOD (b), CAT (c), and POD (d) of *M. officinalis* exposed to Ag NPs

Discussion

The results of the study clearly show the growth-inducing effects of Ag NPs on *M. officinalis*. Growth promotional effects of nanoparticle treatment has been reported in the literature (Pandey et al., 2010; Sharma et al., 2012). The reason for increased growth might be due to ther

increased absorption of inorganic nutrients and photosynthesis rate. Improvement of photosynthetic pigment contents by nanoparticles has been reported by Anusuya and Nibiya Banu, (2016), Priyadarshini et al. (2012), and Siddiqui and Al-Whaibi (2014). These observations indicate that nanoparticles increase the photosynthetic pigment probably by enhancing the electron transport rate (Giraldo et al., 2014).

The content of protein in *M. officinalis* plants correlated with their subsequent growth, showing that the content of endogenous protein, which can be controlled, might be an important factor in subsequent yield of the plant. In accordance with our results, the biosynthesized Ag NPs enhanced protein and carbohydrate contents of *Eichhornia crassipes* (Usha Rani et al., 2016) and *Bacopa monnieri* (Krishnaraj et al., 2012). Furthermore, Anusuya and Banu (2016) reported that chitosan-silver nanoparticles remarkably increased total protein content in chickpea seedlings. The effect of Ag NP treatment on proline is depicted in Fig. (IV. C). Proline contents increased significantly compared with the control by all concentrations of Ag NPs which exhibited time-dependent response. These results are in accordance with the studies showing that proline and soluble sugars accumulate in plants for osmotic adjustment and protection of membrane integrity during abiotic stresses (Jiang et al., 2012; Karimi and Mohsenzadeh, 2017; Vurayai et al., 2011).

Phenols are secondary metabolites of plants comprising phenolic acids, flavonoids, anthocyanins, and tannins. Phenolic compounds have potential protective role against oxidative damage caused by environmental stresses (Kowalska et al., 2014). Few data are available on phenolic acids accumulation by nanoparticles. Our results are in line with the findings of Yasur and Rani (2013), who reported that Ag NPs had positive effects on phenolic content in *Ricinus communis* seedlings. Other studies also showed an increase in total phenol and flavonoid following nanoparticle treatments (Ghorbanpour, 2015; Homaee and Ehsanpour, 2015).

Anthocyanins alleviate stress responses by scavenging free radicals (Hatier and Gould, 2008). Our results concur with a previous report, which showed that the increased anthocyanin content was induced in *Brassica rapa* due to oxidative stress induced by Ag NPs (Baskar et al., 2015). Generally, our results showed increases in total phenolics, flavonoids, and anthocyanin contents under Ag NPs suggesting beneficial effect of Ag NPs in improving scavenging capacity of *M. officinalis* seedlings.

Phenolic acids had been reported to act as antioxidants and metal chelators, reducing lipid peroxidation (Michalak, 2006). For instance, RA is reported to have antioxidant properties and may contribute to the ROS scavenging cascade in plants (Nicolai et al., 2017).

In their study, Yasur and Pathipati Usha Rani (2013) reported that phenolic acids were enhanced in *Ricinus communis* seedlings using Ag NPs. Moreover, it has been reported that application of Ag NPs at proper levels could act as novel elicitors to increase essential oils content (Ghorbanpour, 2015).

The level of MDA is often used as an indicator of reactive oxygen species (ROS) production and the extent of damage occurring to plant in response to oxidative stress (Miller et al., 2010). Our result was in line with Hatami and Ghorbanpour (2014) who reported that increase in SOD activity, accompanied by increase in CAT and POX, could be considered as an efficient strategy to reduce oxidative damage. The maximum induction of antioxidant enzyme activities (day 15) were detected along with a decrease in MDA, suggesting that Ag NPs reduced oxidative damage by increasing the activities of SOD, CAT, and POX. Enzyme activity may change depending on the concentration, exposure time, and type of nanoparticles (Movafeghi et al., 2018). Our results are in accordance with the results of Priyadarshini et al. (2012), who reported that Ag NPs decreased ROS accumulation by increasing the activity of antioxidant enzymes.

Conclusions

In the present study, we evaluated time course responses of *M. officinalis* to various concentrations of Ag NPs. We found that biologically synthesized Ag NPs were acting as a

growth promoter and 60 ppm Ag NPs concentration on day 15 was optimum for inducing the growth promotional response in *M. officinalis*. Results are also supported by the measurements of plant weight, photosynthetic pigment, etc. Increase in the proline and phenolic contents and also up-regulation of oxidative stress-related enzymes showed the activation of plants' antioxidant defense mechanisms to decrease lipid peroxidation. Our results for the first time suggest that Ag NPs could act as an elicitor to induce favorable metabolites and physiological responses and could be a promising strategy in the field of secondary metabolites production of *M. officinalis* plant.

Acknowledgements

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