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Assessing the toxicity of silver nanoparticles in cell suspension culture of *Nicotiana tabacum*

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ABSTRACT

Silver nanoparticles (Ag NPs) are one of the commercialized nanomaterials that are involved in quite a lot of industrial and biomedical utilization. Although some efforts have been made to examine the possible toxicity of Ag NPs on plant systems, but no consensus has been reached on this issue. To determine the effect of Ag NPs on antioxidant defense system in cell suspension cultures of *Nicotiana tabacum*, we evaluated the changes in multiple biomarkers of oxidative stress. Ag NPs were synthesized using photo reduction method and characterized as NPs with spherical shape and diameters in the range of 10-100 nm by means of XRD, DLS, and SEM techniques. A significant decline in the cell growth and viability even at low concentration of Ag NPs (5 mg L^{-1}) were evident. Interestingly, a progressive decline in hydrogen peroxide levels was observed, while the contents of malondialdehyde did not change. The activity of ROS scavenging enzymes augmented with the increasing concentration of Ag NPs. Additionally, the contents of phenolic and flavonoid contents were reduced after exposure of the cells to higher concentrations (15, 20, and 25 mg L^{-1}) of Ag NPs. These findings confirmed that Ag NPs triggered oxidative stress in cell suspension culture of *N. tabacum*.

Keywords: *Silver nanoparticles; Nicotiana tabacum; cell suspension cultures; antioxidant defense.*

1. INTRODUCTION

Silver nanoparticles (Ag NPs) are one of the commercialized nanomaterials that are involved in several industrial and biomedical applications. Due to their special properties of silver and nano size, they have been used in many personal care products, food services, optical sensors, medical instruments, textiles, drug delivery systems, and as anticancer agents [1, 2]. They also possess excellent antimicrobial and anti-inflammatory activities that make them promising agents for potential biomedical applications [3, 4]. Therefore, a number of physical, chemical, and biological techniques have been employed for the synthesis of Ag NPs [5, 6].

The disadvantage of Ag NPs is the induced toxicity in biological systems at different degrees. A number of studies have been shown that Ag NPs can cause adverse effects on humans as well as the environment [7, 8]. Since plants are the basic components of ecosystems, the phytotoxic effects of Ag NPs should be given particular emphasis in toxicological studies. Heretofore, a number of plant-based experimental systems have been used to evaluate the impacts of Ag NPs on plants. Studies on the growth of plants in hydroponic media revealed the phytotoxicity of Ag NPs and confirmed their uptake, translocation, and accumulation in different organs [9-12]. In addition, solid growth matrices have been utilized in Ag toxicity studies [13]. For example, investigation on the impact of commercial Ag NPs on *Triticum aestivum* in sand indicated the translocation of Ag from exposed roots into shoots [14]. Nano forms of Ag also significantly inhibited the development of *Capsicum annuum* grown in a commercial substrate [15]. There are some evidence showing the significant impact of the utilized medium for toxicological studies on phytotoxicity of Ag NPs. Assessment of the effects of silver ions dissolved from Ag NPs on crop plant species indicated that they were less toxic in soil than agar solidified media [16]. Beside the whole-plant trials, *in vitro* plant cell and tissue cultures have been exploited in different studies intended for recognizing the interaction of plant cells with a wild variety of organic and heavy metal pollutants [17, 18] as well as NPs [19, 20]. Practical convenience and technical advantages of these methods, including eliminating the effects of microflora and translocation barriers, make them useful experimental models for toxicity and tolerance studies [21].

Although Ag NPs provide many benefits, various aspects of their toxicity have remained unknown. Hence, further toxicological investigations seem to be substantial for understanding the association of the physicochemical properties of Ag NPs with their toxicity. The current study was designed to evaluate the toxicity of Ag NPs in cell suspension cultures of *Nicotiana tabacum*, as a model system for the research of NPs stresses [22-24]. So far, no previous research has examined the toxic impacts of Ag NPs on tobacco cells despite the fact that these cell cultures are used extensively in plant science. In the current study, we considered some factors such as growth and cell viability; ROS level; superoxide dismutase (SOD, EC 1.15.1.1) as well as peroxidase (POD EC 1.11.1.7) activity; and content of MDA, total phenols and flavonoids to conclude whether Ag NPs can induce oxidative stress in tobacco cell cultures.

2. EXPERIMENTAL SECTION

2.1. Synthesis and characterization of Ag NPs. In order to the synthesis of Ag NPs by using photo reduction method, 1.6 g of polyethylene glycol (2000) was dissolved in 20 ml of ethanol (96%) and then added to the $AgNO_3$ solution (0.4 g in 5 ml of deionized water). The solution was refluxed for 3 h. The mixture was stirred for 2 h under UV-C (30W) lamp to reduce Ag^+ to Ag nanoparticles. The precipitate was separated from the solution by centrifugation at 8000 g for 5 min, washed with deionized water repeatedly to remove the residual Ag^+ and then placed in an autoclave at 121 °C for 20 minutes.

The synthesized Ag NPs were characterized using field emission scanning electron microscope (FESEM) (CamScan mv2300), X-ray powder diffraction (XRD) (ADVANCE D8, Bruker, Germany), and dynamic light scattering (DLS) (Zetasizer Nano-ZS, Malvern Instruments, UK) techniques.

2.2. Preparing tobacco cell suspension cultures. Cell suspension cultures were obtained from three-month-old calli, (initiated from cotyledon explants of 3-week-old seedlings of *N. tabacum* cv. Virginia). The cells were cultured in the Murashige and Skoog (MS) medium supplemented with 2 % sucrose and 1 mg L^{-1} of 2,4-dichlorophenoxyacetic acid. The cells were subcultured once a week and maintained under constant shaking (110 rpm) at 25 °C in the dark. For the Ag NPs toxicity analysis, the experiments were conducted in the exponential phase of growth (4-day-old cell culture). The cells were exposed to different concentrations of Ag NPs (0, 5, 10, 15, 20, and 25 mg L^{-1}) and were harvested after 48 hours (h) of incubation for different assays. Dry weight of the samples was measured after drying in an oven at 50 °C for 24 h and was expressed as mg per flask, where the total concentration of cultures was 10 ml.

2.3. TTC assay. Cell viability was assessed using triphenyl-tetrazolium chloride (TTC) assay [25]. Percentage of cell viability was calculated according to the optical density (OD) at 485 nm (SPEKOL 1500 UV-VIS spectrophotometer) of treated tobacco cells with Ag NPs compared with control.

2.4. Microscopic observations. Neutral red (NR) staining was performed according to method reported by Kariya et al. [26] in order to determine the cytotoxicity in the cells. Moreover, the effect of Ag NPs on tobacco cells apoptosis were determined morphologically by staining with 5 µg ml^{-1} acridine orange (AO), for 30 min at the dark and fluorescence microscopy (Novel N800-F).

2.5. Evaluation the content of hydrogen peroxide. Assessing the hydrogen peroxide (H_2O_2) levels was done based on the method reported by Velikova et al. [27]. The absorbance of the experiments was read at 390 nm and H_2O_2 concentration was calculated using a standard curve and expressed as µmol g^{-1} FW.

2.6. MDA assay. For the determination of lipid peroxidation, malondialdehyde (MDA) content was measured [28]. The absorbance of the reaction mixture was detected at 532 nm and the MDA concentration was quantified based on 3, 1, 1, 3-tetraethoxy propane (0–100 nM) standard curve and expressed as nmol g^{-1} FW.

2.7. Total protein content and antioxidant enzyme activity. An amount of 0.2 g of fresh tobacco cells was homogenized in pre-chilled mortar and pestle with 1 mL of 50 mM potassium phosphate buffer (pH 7). The homogenate was centrifuged at 10,000 g at 4 °C for 20 min. The resulting supernatant was used to measure the antioxidant enzyme activities. Total SOD activity was measured according to the method described by Winterbourn et al. [29]. The quantity of enzyme required for the inhibition of 50% of nitro blue tetrazolium chloride reduction was defined as one unit of SOD activity. Total POD activity was quantified by the technique reported by Chance and Maehly [30]. POD activity was considered as the enzyme amount capable of converting 1 µmol guaiacol to tetraguaiacol per minute per ml. The content of total soluble protein was calculated using Bradford method [31] and expressed as mg g^{-1} FW.

2.8. Total phenolic and flavonoid contents. Phenolic and flavonoid contents, extracted with methanol (0.3 g tobacco cells per 1 mL), was quantified using the Folin-Ciocalteu method [32] with gallic acid as the standard and the method of Quitter-Deleu et al. [33] with quercetin as the standard, respectively. Their quantities were expressed as µg g^{-1} FW.

2.9. Statistical analysis. The data were subjected to one-way analysis of variance (ANOVA; SPSS 22 for Windows) followed by Duncan's test ($P \leq 0.05$). The measurements were conducted in triplets and the obtained results were expressed as the mean ± SD.

3. RESULTS SECTION

3.1. Characteristics of Ag NPs. Synthesized Ag NPs was characterized using different techniques. Based on SEM analysis, the shape of synthesized Ag NPs were determined as spherical with diameters ranging of 10-100 nm (Figure 1). The XRD pattern confirmed the crystalline nature of synthesized Ag NPs (Figure 2). XRD peaks at 2θ of 38.22°, 44.38°, 64.48°, and 77.38° could be attributed to the 111, 200, 220, and 311 crystallographic planes of face centered cubic crystal structure of Ag [34]. The size distribution profile of NPs in suspension as well as zeta potential were determined by DLS as 22.95 nm and -15.4 mV, respectively (Figure 3 a, b). The negative value confirms the high disparity and stability of Ag NPs [35]. According to these results the characteristics of the synthesized Ag NPs seemed to be appropriate for uptake by cells of *N. tabacum*.

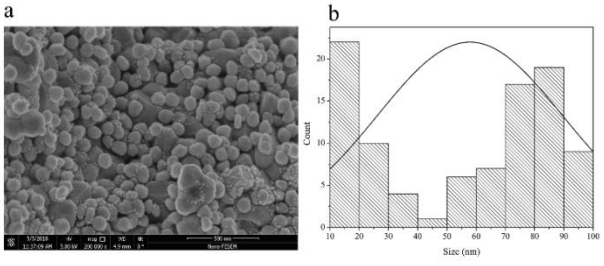

Figure 1. SEM image (a) and size distribution (b) of Ag NPs.

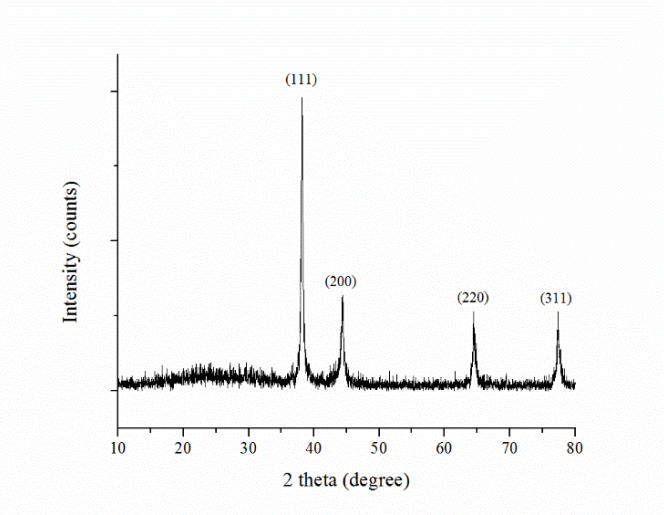

Figure 2. XRD pattern of Ag NPs.

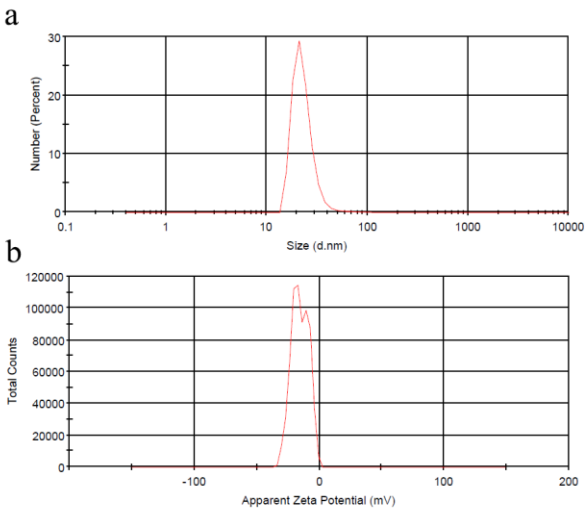

Figure 3. DLS measurements. Particle size distribution (a) and zeta potential (b) of Ag NPS.

3.2. Effects of Ag NPs on cell viability and growth. Viability assays are one of the essential steps in toxicity investigations that demonstrate the cellular reaction to a toxicant. The influences of Ag NPs on the TTC reductions in tobacco cells are shown in Figure 4, a. A progressive decline in viability observed when cells were treated with Ag NPs after 48 h, which was approximately 95, 19, 11, 2 and 3 % for 5, 10, 15, 20 and 25 mg L^{-1} of Ag NPs, respectively. Actually, TTC assay not only gives information on the cell survival, but also indicates metabolic activities of cells. Hence, it can be concluded that dehydrogenases have either denatured or degraded after exposure of tobacco cells to different concentrations of Ag NPs. In the same way, growth parameters were changed by Ag NPs treatments. The growth was noticeably inhibited at the applied concentrations of Ag NPs (Figure 4 b). In the case of the lowest and the highest concentrations (5 and 25 mg L^{-1}), the final dry weight was 72 and 44 % compared to the control.

Neutral red (NR) is a vital stain and preferentially stored as a protonated form with red color inside the vacuoles of living cells. Accordingly, NR staining was conducted in order to determine the cytotoxic impacts of Ag NPs on tobacco cells. Microscopic observation showed that most control cells had light red color, indicating one large vacuole with low pH (Figure 5 a). Conversely, the cells treated with 10 mg L^{-1} of Ag NPs exhibited different stained cells (Figure 5 b). The vacuoles of some cells were heavily stained, while others showed bright red color and even there were cells without color. Furthermore, the treatment with 25 mg L^{-1} of Ag NPs induced cytotoxicity with cells carrying high NR stain and cells with a stained nucleus (Figure 5 c). It could be concluded that lack of NR uptake was owing to loss of cell viability. In addition, high accumulation of NR in the treated cells indicated that the vacuoles were still alive and fight against toxicity of Ag NPs. Actually, transport of toxic metals into the vacuole is one of the ways of reducing the effects of toxic metals in plant cells [36]. Likewise, accumulation of Ag NPs in vacuoles has been previously reported [37].

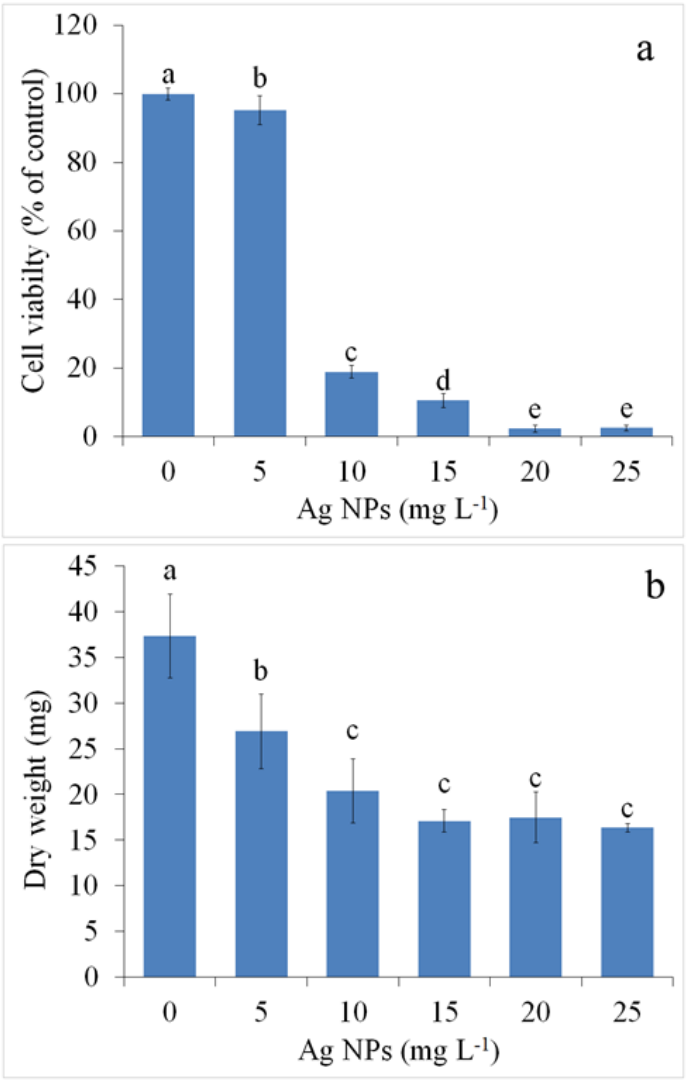

Figure 4. Effect of Ag NPs on the viability (a) and growth (b) of the tobacco cells.

Programmed cells death was recognized by acridine orange (AO) staining as morphological changes in nuclei of the tobacco cells after exposure to 10 and 25 mg L^{-1} of Ag NPs. A number of nuclear apoptotic bodies enclosing the fragmented DNA were observed in the cells after 48 h exposure to 10 mg L^{-1} of Ag NPs indicating early apoptosis in the cells (Figure 5 e, white arrow). However, 25 mg L^{-1} of Ag NPs caused drastic changes in nuclei with intensely fragmented chromatin that confirm late apoptosis in the tobacco cells (Figure 5 f, white arrow), in comparison to the nuclei of control cells that was visualized as regular spherical units

(Figure 5 d). In fair agreement with these findings the impact of Ag NPs in induction of genotoxicity is already reported in plant systems [38, 39].

3.3. Antioxidative status of tobacco cells. At the lowest concentration of Ag NPs (5 mg L^{-1}) an increase in H_2O_2 level were observed, but it declined then significantly at 10, 15, 20 and 25 mg L^{-1} of Ag NPs (Figure 6 a). Formation of reactive oxygen species (ROS) is a mechanism, by which heavy metals and NPs cause oxidative damages in plant cells [40, 41]. ROS generation and elevation of H_2O_2 content has been reported in some plant species such as *Allium cepa* [42]; *Vigna radiate* [43]; *Solanum tuberosum* [44]; and *Spirodela polyrhiza* [45] after exposure to Ag NPs. On the contrary, a significant reduction in H_2O_2 accumulation has been described in Ag NPs-treated *Brassica juncea* seedlings that is in accordance with our results [46]. Reduction in H_2O_2 concentration in plants under CeO_2 NPs stress was reported to be on account of the radical scavenging ability of CeO_2 [47]. As well, Ag NPs are potential catalysts that can act as acceptor and donor of electrons in a redox reaction [48, 49]. However, more assessments are required to shed light on the responsible mechanism for triggering the redox response in plants by Ag NPs.

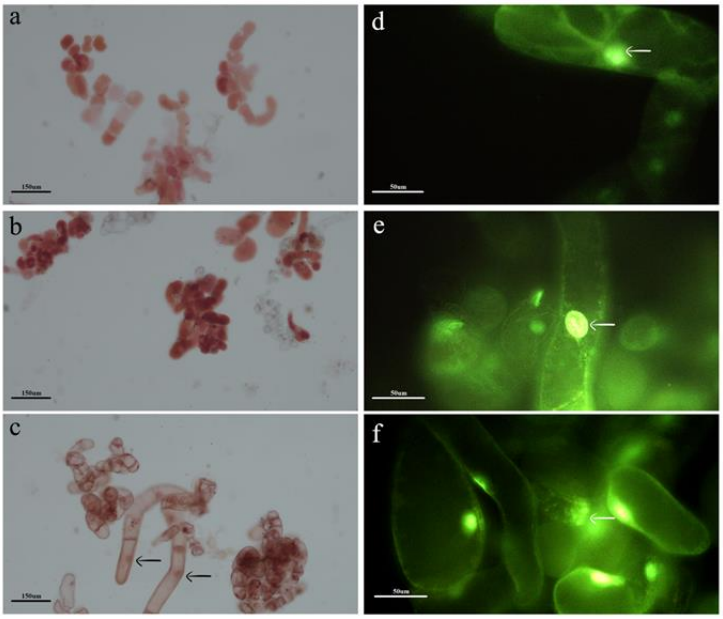

Figure 5. Light microscopy images of vacuoles after vital staining with NR (a, b, c) and fluorescence microscopy images of nuclei after staining with AO (d, e, f). Several types of NR-stained cells were observed in Ag NPs treated cells: control cells with light red color (a); treated cells by 10 mg L^{-1} of Ag NPs with deep red colored and light red colored vacuoles (b); the cells after the exposure to 25 mg L^{-1} of Ag NPs with deep-red colored vacuoles and stained nucleus (c, shown with black arrows). AO staining morphologically showed programmed cells death; nucleus of control cell (d); DNA fragmentation in cells treated by 10 mg L^{-1} of Ag NPs (e); late apoptosis with intensely fragmented chromatin in the cells treated by 25 mg L^{-1} of Ag NPs (f).

Analysis of the effect of Ag NPs on lipid peroxidation in tobacco suspension cultures during a growth period of 48 h, showed no significant effect of the increasing concentration of Ag NPs on the MDA content of the cells (Figure 6 b). Indeed, lipid peroxidation is a biological indicator for the ROS mediated damage as it was observed in most Ag NPs toxicity studies in plants [45, 50, 51]. In view of radical scavenging ability of Ag NPs as well as boost in antioxidant enzymes activity, it can be concluded that the reduced levels of H_2O_2 avoided the possible lipid damage affected by the Ag NPs. Our results are in agreement with the previous observations of the influence of CeO_2 NPs on MDA concentrations in *Zea mays* [52].

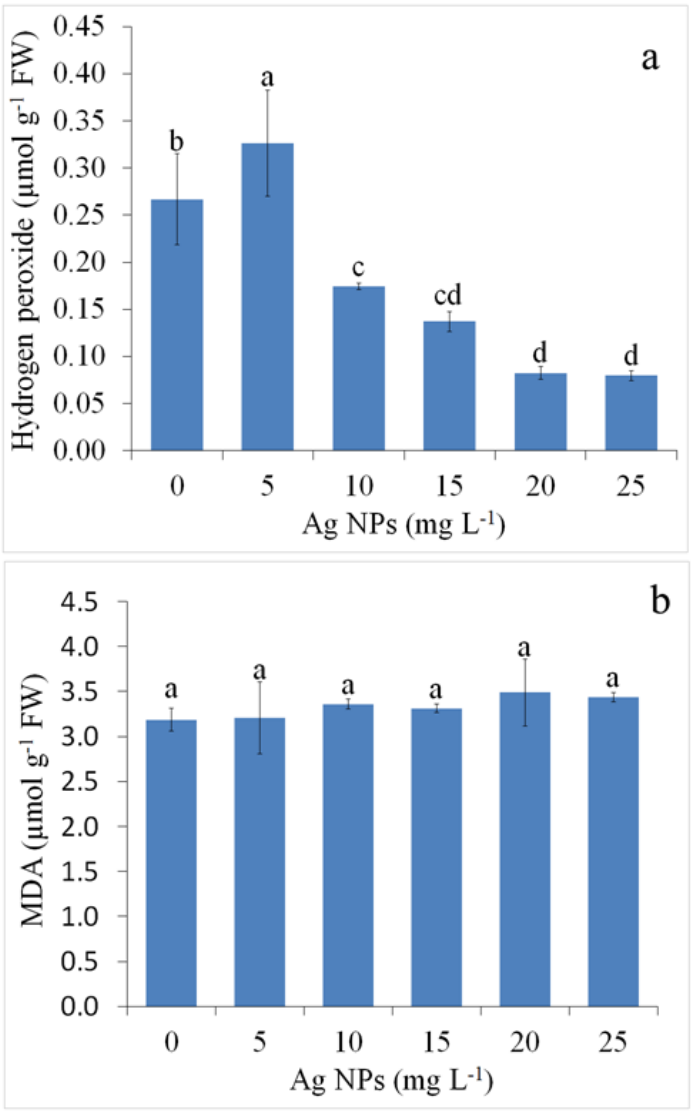

Figure 6. Generation of hydrogen peroxide (a) and MDA (b) caused by treatment with Ag NPs.

3.4. Activity of antioxidant enzymes. Total protein content as well as SOD and POD activities were assessed in tobacco cells after 48 h of exposure to different concentrations of Ag NPs (0, 5, 10, 15, 20 and 25 mg L^{-1}). Protein content was significantly decreased in cells treated with Ag NPs compared to control (Figure 7 a). Besides, Ag NPs caused a heightening in the activity of SOD and POD as the most important ROS-scavenging enzymes (Figure 7 b, c). A raised activity of SOD was detected in 10 (1.6%), 15 (2.2%), 20 (3.3%), and 25 mg L^{-1} (4.9%). Correspondingly, 10, 15, 20 and 25 mg L^{-1} of Ag treatment led to approximately 2, 3, 4 and 5 times higher POD activity.

Similar to our observations, the protein content in different organs of *Spirodela polyrhiza* and *Bacopa monnieri* treated with Ag NPs were lower than the content in control plants [53]. In the same way, treatment of tomato plants with Ag NPs instigated a notable increase in protein degradation, resulting in accumulation of amino acids [54]. As a result, a high concentration of Ag NPs diminished protein biosynthesis or enhanced protein decomposition because of oxidation [45].

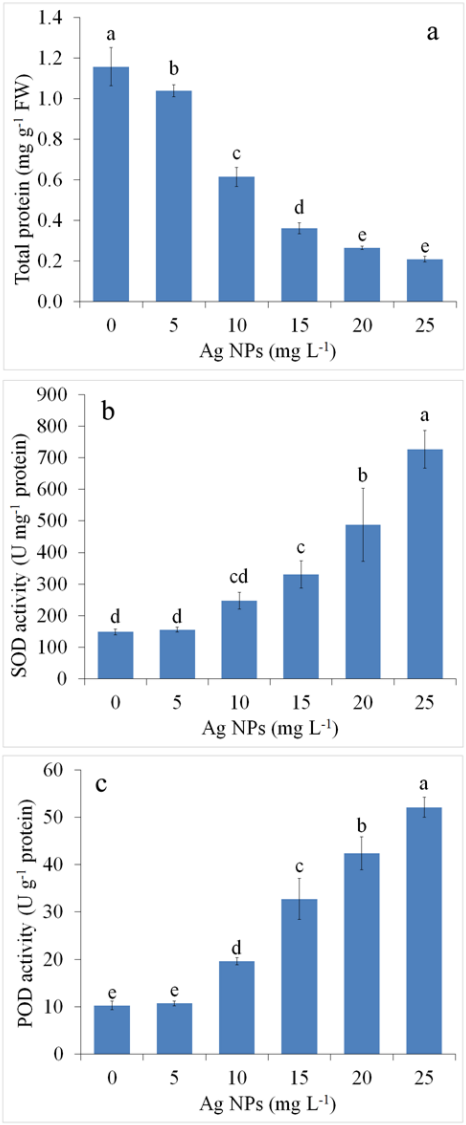

Figure 7. Changes in total protein (a) and the activity of SOD (b) and POD (c) antioxidant enzymes in tobacco cells treated with Ag NPs.

The response of antioxidant enzymes to NPs stress varies noticeably depending on plant species, stage of development, and nature of NPs [55, 56]. Our results showed that both SOD and POD were at their maximum activity levels at 25 mg L^{-1} of NPs. Recently, changes in antioxidant defense components have been examined as valuable biomarkers in ecotoxicological trials using Ag NPs. Rises in total SOD activity were detected following the exposure of *Ricinus communis* to Ag NPs [57]. An increased SOD activity in *Lycopersicon esculentum* plants treated with Ag was also reported [58]. The enhanced activity of SOD and POD indicated that these enzymes were initiated after Ag NP exposure and helped to scavenge the ROS in aquatic plant *S. polyrhiza* [45]. Elevated levels of POD activity were recorded in *B. monnieri* subjected to biologically synthesized Ag NPs treatment [53]. These findings confirm the results of gene expression patterns in terms of up-regulation of SOD and POD enzymes [43, 50, 59]. Accordingly, different plants tolerate environmental stresses by stimulation in the activities of antioxidative enzymes.

3.5. Phenolic and flavonoid contents. The amount of phenols was not affected at the 5 and 10 mg L^{-1} of Ag NPs, but significantly declined in the cells treated with Ag NPs at 15, 20 and 25 mg L^{-1} compared to the control (Figure 8 a). Furthermore, flavonoid content was reduced after Ag NPs treatments of cells with 10 - 25 mg L^{-1} for 48 h (Figure 8 b). Beside enzymatic system, plant cells have a nonenzymatic antioxidative defense system against environmental stresses, which mainly composed of phenols and flavonoids [60]. Phenolic compounds can be oxidized by guaiacol type peroxidases for detoxification of H_2O_2, directly scavenge ROS, or act as metal chelators [61]. Remarkably, our results showed significant drop in total phenolic and flavonoid contents in tobacco cells despite the oxidative stress resulting from the exposure to Ag NPs. These findings are in contrary to the reports that showed accumulation of phenols in response to the Ag NPs-mediated oxidative stress [53, 57], but in agreement with reduction in antioxidant activity by increasing magnetic NPs concentration in tobacco BY-2 cells [22]. Likewise, CeO_2 NPs at the concentration of 800 mg kg^{-1} significantly decreased the phenolic content in cucumber plants [62]. Moreover, the CeO_2 reduced the capacity of antioxidants such as phenols in rice grains [63]. We assume that the reductions in the total phenolic and flavonoid contents point out the antioxidative activity of tobacco cells under Ag NPs stress because they show a protective role by acting as metal chelators and ROS scavengers.

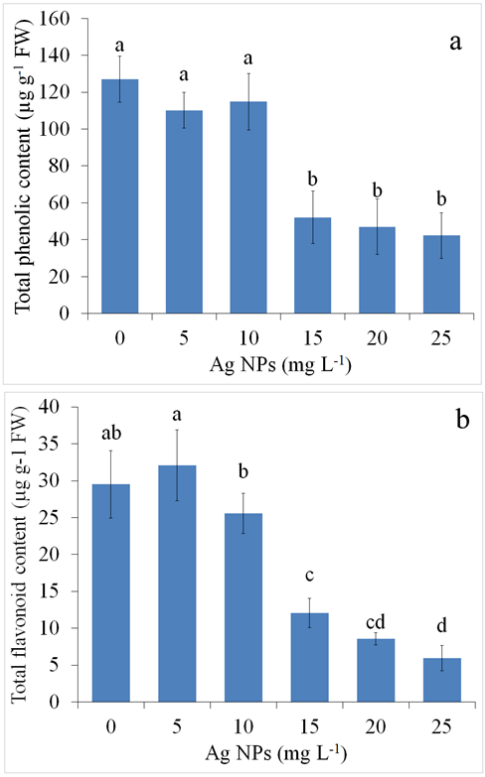

Figure 8. Total content of phenolics (a) and flavonoids (b) in tobacco cells after treatment with Ag NPs.

4. CONCLUSIONS

Application of Ag NPs in different industrial and biomedical areas has been gained extensive attention, and thus more awareness about their impact on the environment and living organisms is required for safety assessments and risk management. Ag NPs significantly decreased growth and viability of tobacco cells in all applied concentrations of Ag NPs. Increasing activities of scavenging antioxidant enzymes and lessening content of non-enzymatic antioxidants indicated that Ag NPs induced oxidative

stress on tobacco suspension cultures. There are controversial information about the induction of hydrogen peroxide by Ag NPs in different examined plant systems. Reduction in hydrogen peroxide levels by the increasing concentrations of NPs may possibly be owing to the radical scavenging ability of Ag NPs. However, application of diverse plant-based experimental systems makes it difficult to compare the results of toxicity studies. Therefore, comparative studies on Ag NPs in different plant-based experimental systems are essential to clarify this issue in details.

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