Iran J Sci Technol Trans Sci (2020) 44:621–627
https://doi.org/10.1007/s40995-020-00878-4

RESEARCH PAPER

Effect of Silver Nanoparticles on Production of Indole Alkaloids in *Isatis constricta*

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Received: 11 February 2020 / Accepted: 27 April 2020 / Published online: 22 May 2020

Abstract
Silver nanoparticles (AgNPs) are widely used in many applications of biotechnology, including medicine and agriculture. They are released to the nature as waste materials, which can cause physiological and biochemical effects on plants. Indigo, indirubin and tryptanthrin are valuable indole alkaloid compounds in *Isatis constricta* due to both medicinal effects and dye properties. This research was conducted to determine the effects of different concentrations (0, 0.25, 0.5, 1, 1.5 and 2 mg L^{-1}) of AgNPs on the production of indigo, indirubin and tryptanthrin compounds in leaves of in vitro grown shoots of *I. constricta* Davis. Indigo production was 1.15-fold of control (869 ± 8.33 µg g^{-1}) in the leaves of shoots regenerated in Murashige and Skoog supplemented with 2 mg L^{-1} of AgNPs (1003 ± 11.42 µg g^{-1}) on 5 days post-treatment. Tryptanthrin production showed an increase in all applications of AgNPs, but the highest increase was observed at a concentration of 2 mg L^{-1} (4.59 ± 0.046 µg g^{-1}) and this increase was 1.71-fold of control (2.68 ± 0.031 µg g^{-1}) on 5 days post-treatment. The production of indigo and tryptanthrin decreased on 10 and 15 days post-treatment with AgNPs. The contents of indirubin decreased during day 5-10-15 and at all concentrations of AgNPs compared to the control.

Keywords Tryptanthrin · Indirubin · Indigo · AgNPs · *Isatis constricta*

1 Introduction

Plants are important sources of secondary metabolites. Major roles of these metabolites are to protect plants against various biotic and abiotic stresses. Many of the secondary metabolites synthesized by plants are used in drug industry and as source of dyestuff in textile industries (Guerriero et al. 2018), but their quantities are generally very low (Bourgaud et al. 2001). These secondary metabolites are accumulated in plants when subjected to various signal molecules and chemical elicitors (Ramakrishna and Ravishankar 2011).

Plant tissue culture has been used to enhance the production of many valuable compounds which are synthesized in small amounts by plants but of great commercial importance (Zhao et al. 2005; Abouzid 2014). In order to increase secondary metabolite production in plants, it may be effective to apply various precursors, elicitors, heavy metals and nanoparticles to plants grown in vitro (Giri and Zaheer 2016).

Nanoparticles are substances that are less than 100 nm and have unique physical and chemical properties (Yan and Chen 2019). Nanoparticles have different effects on plants depending on the concentrations, properties and exposure time of the nanoparticles (Stampoulis et al. 2009). Among the nanoparticles, silver nanoparticles are the ones of the most using due to antiseptic, antibacterial and antifungal properties, as well as in household products, textiles and medical devices (Duran et al. 2007; Nelson et al. 2007; Tran et al. 2013). In agricultural applications, nanoparticles have important roles, especially in minimizing the use of chemical fertilizers, and improve growth and yield of crops (Siddiqui and Al-Whaibi 2014). Besides, AgNPs have been used influencing plant cell growth, biomass production and production of some valuable secondary metabolites in in vitro cultures (Elechiguerra et al. 2005).

I. constricta Davis is a biennial member of genus *Isatis* (Cruciferae) and an endemic to the South-Eastern Anatolia Region of Turkey (Mısırdalı 1985). Species belonging to

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genus *Isatis* have been widely cultivated in China for features of medicinal and dye. Particularly, *I. tinctoria* and *I. indigotica* have been used in modern and traditional medicine for the treatment of hepatitis, viral pneumonia, influenza, snake bites, hemorrhoids and various inflammatory diseases (Chang et al. 2012; Hamburger 2002). Diverse bioactive constituents have been reported from methanol extracts of leaves and roots of the *Isatis* species (Liau et al. 2007; Karakas 2019). Alkaloids, which are used in both treatments and source of dyestuff, are evaluated as characteristic compounds of these plants (Bektas et al. 2016). Up to now, more than 100 alkaloids have been identified in *Isatis* species, such as quinoline alkaloids, quinazoline alkaloids and indole alkaloids (Zhang et al. 2019). Particularly, the leaves of these plants are used as a source of dark blue dye (indigo) for thousands of years in China and for centuries in Europe (Puchalska et al. 2004). In addition to be a source of indigo dye, numerous studies were carried out on *Isatis* plants due to their bioactive constituents that have numerous pharmacological effects. Indirubin and tryptanthrin, which are also important indole alkaloids obtained from *Isatis*, have therapeutic and dye properties (Honda et al. 1980; Mohn et al. 2009; Namgung et al. 2019). Moreover, tryptanthrin is a yellow, and indirubin is a red-colored compound. In terms of medicinal effects, tryptanthrin has anti-inflammatory, antibacterial and anticancer effects (Jahng 2013; Kaur et al. 2017). In addition to the dye properties, due to its biological and pharmaceutical activities, indirubin and its derivatives have been used for the treatment of type 2 diabetes (Bertrand et al. 2003), Down syndrome, Alzheimer's (Myrianthopoulos et al. 2013) and chronic myelocytic leukemia (CML) (Kim et al. 2013).

In this research, it was aimed to investigate the effects of silver nanoparticles (AgNPs) on the production of indigo, indirubin and tryptanthrin in the leaf extracts of *I. constricta* Davis grown under in vitro conditions.

2 Experimental

2.1 Plant Material and *In Vitro* Culture Conditions

The seeds of *I. constricta* were collected in June 2017 from Diyarbakır District, Ergani Province (942 m above sea level), Turkey. The samples of the plant were identified by Prof. Dr. Ömer SAYA from Dicle University, Science Faculty, Department of Biology. The mature seeds were used as initial material. Firstly, the seeds of plant were washed in tap water for 5 min and surface-sterilized by submerging in a 70% ethanol solution for 60 s, followed by immersion in a 10% sodium hypochlorite (NaOCl) for 6 min, and then rinsed with sterile distilled water five times. The sterilized seeds were inoculated in hormone-free MS solid medium (Murashige and Skoog 1962)[27] that contained 0.6% agar and 3% sucrose. The pH of medium was adjusted to 5.8 with 2 N NaOH prior to autoclaving at 1 atm, 121 °C for 20 min. Later on, the cultures were incubated under a photoperiod of 16/8 h light and darkness in a growth chamber at 25 ± 2 °C. After 3 weeks initiation of cultures, plantlets were subcultured in hormone-free solid MS basal medium containing 0.6% agar and 3% sucrose for plant proliferation (Karakas 2019).

2.2 Treatment with AgNPs

Before being added to the culture medium, AgNPs were exposed to ultrasonication for 10 min followed by filtration (pore size of 0.45 μm). Micropropagated plantlets at the end of third subculture were transferred to a solid MS medium containing different concentrations of AgNPs (0, 0.25, 0.5, 1, 1.5 and 2 mg L^{-1}). The application of nanoparticles was maintained for 15 days. On day 5-10-15 of AgNPs application, plantlets were harvested for determining the effects of AgNPs on the production of indigo, indirubin and tryptanthrin in the leaf explants.

2.3 Reagents and Chemicals

Silver nanoparticles (AgNPs, 99.9%) were purchased from Gute Chemie-abcr Gmbh, Deutsch. Standard compounds of indigo (≥ 98%), indirubin (≥ 98%) and tryptanthrin (≥ 98%) were purchased from Sigma (St. Louis, Mo, USA). *N,N*-Dimethylformamide (DMF, ≥ 99.9%), acetonitrile (ACN, ≥ 99.9%) and methanol (≥ 99.9%) were purchased from Merck (Darmstadt, Germany), and trifluoroacetic acid (TFA, ≥ 99%) was purchased from Merck (Hohenbrunn, Germany). ACN and MeOH in HPLC grade purity and double distilled water were used.

2.3.1 Calibration Curves of Tryptanthrin and Indirubin

Standards of indigo, indirubin and tryptanthrin were dissolved in *N,N*-dimethylformamide (DMF). Each standard solution of indigo, indirubin and tryptanthrin was prepared at seven different concentrations (0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 mg L^{-1}) by diluting their stock solutions (1 mg mL^{-1}) with DMF. To generate calibration curves, each standard solution was injected in triplicate to high-pressure liquid chromatography (HPLC). The concentrations of indigo, indirubin and tryptanthrin in the extracts were calculated using calibration curves of the compounds (Fig. 1a–c).

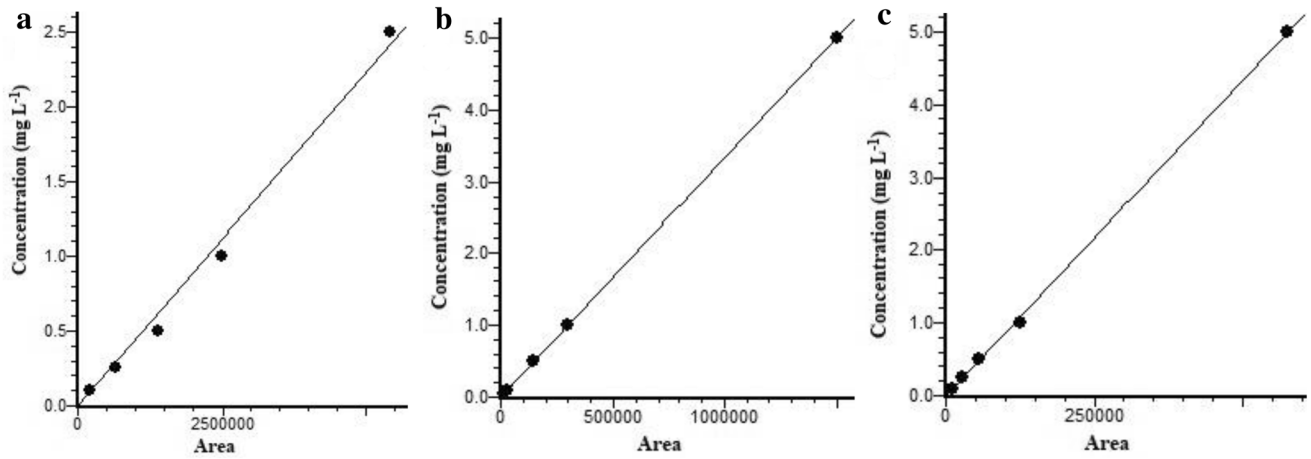

Fig. 1 Calibration curves of **a** indigo, **b** indirubin, **c** tryptanthrin

2.4 Extraction of Indigo, Indirubin and Tryptanthrin

The protocol developed by Liau et al. (2007) was used for the extraction of indole alkaloids. Briefly, the leaf explants of plantlets were powdered using a laboratory blender. A quantity of 200 mg of air-dried leaf samples were accurately weighed and exposed to sonication (Jeiotech, US-05, Korea) in 10 ml of MeOH for 5 min at 48 °C. These processes were carried out in triplicate (10 mL × 3). After sonication, methanolic parts obtained were combined in a 50-mL flask, and MeOH in the flasks was removed in vacuo (LabTech EV311, Evaporator). The final samples were mixed with MeOH/DMF (2.5:2.5) of 5 mL, and the final volume was adjusted to 40 mL with MeOH. The solution was filtered through a 0.45-mm nylon filter membrane (Merck Millipore® syringe filter) prior to analysis.

2.5 Analysis of Indigo, Indirubin and Tryptanthrin Using HPLC

HPLC analysis was performed using an autosampler (SIL 20A-HT) injecting 20 µL of each sample, a binary pump (LC-20AT) solvent delivery system working at a flow rate of 0.5 mL min^{-1}, a dual-wavelength absorbance detector (SPD M-20A PDA) and degasser (DGU 20A5R). The column, Inertsil ODS-3 (GL Sciences Inc., Japan) with 5 µm × 4.6 mm × 250 mm in length and 5 µm particle size, was kept warm at 30 °C in a column oven system (CTO-10AS VP SHIMADZU). Isocratic flow was performed using CH_3CN/H_2O 65/35 with 0.1% TFA, running 20 min.

Separation process of three compounds was performed at room temperature. The mobile phase was the same for indigo, indirubin and tryptanthrin, but the wavelengths were different (indigo 279 nm, indirubin 275 nm and tryptanthrin 305 nm). Retention times of indigo, indirubin and tryptanthrin were 16.2, 11.4 and 15.2 min, respectively (Fig. 2a–c). The correlation coefficients (R) of the standard compounds were 0.9999 for indigo and tryptanthrin; it was 0.9997 for indirubin. Quantification of the three compounds was carried out by comparing the retention time of the standard compounds.

2.6 Statistical Analysis

All experiments were done as three replicates for the determination of changes in amount of each alkaloid depending on application time and different concentrations of AgNPs. Analysis of variance (ANOVA) was performed using SPSS Software Version 16.0 for Windows. The means were compared using DUNCAN's at $p \leq 0.05$ level of significance as the mean ± standard deviation.

3 Results and Discussion

3.1 Influences of AgNPs on the Production of Indigo, Indirubin and Tryptanthrin

In the present study, effects of different concentrations of AgNPs (0.25, 0.5, 1, 1.5 and 2 mg L^{-1}) were investigated on indigo, indirubin and tryptanthrin production in the leaf explants of *I. constricta* grown under in vitro conditions for 15 days. HPLC chromatogram of leaf extracts was obtained according to retention times of standard compounds (Fig. 3a–c).

Figure 4a–c shows the effects of AgNPs on indole alkaloids (indigo, indirubin and tryptanthrin) production in the leaves of *I. constricta* grown in vitro. The treatment with different concentrations of AgNPs to plantlets showed

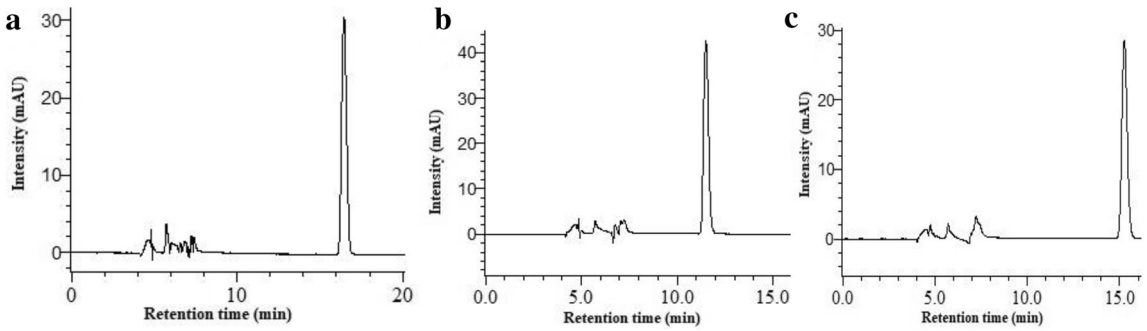

Fig. 2 HPLC chromatogram for the standard compounds of **a** indigo, **b** indirubin, **c** tryptanthrin

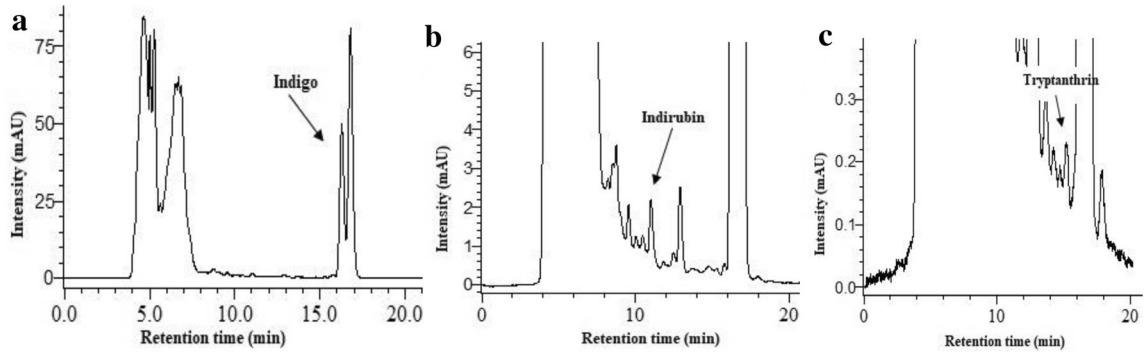

Fig. 3 HPLC chromatogram of the leaf extracts treated with 2 mg L^{-1} of AgNPs. **a** Indigo. **b** Indirubin. **c** Tryptanthrin

significant effects on indigo, indirubin and tryptanthrin production. Treatments of AgNPs at concentrations between 0.25 and 1.5 mg L^{-1} and production of indigo exhibited no effect, but at a concentration of 2 mg L^{-1} (1003 μg g^{-1}), it was 1.15-fold of control (869 ± 7.50 μg g^{-1}) in plantlets harvested on the day 5 of experiment. On day 10 and 15 of AgNPs application, production of indigo showed a significant decrease at all concentrations and the highest decrease was observed in the leaf explants treated with 2 mg L^{-1} AgNPs (382 ± 0.76 μg g^{-1}) on day 15, about 2.2-fold of control. The production of indirubin was low during the experiment, at the three periods (on day 5, 10 and 15 post-treatment) and at all concentrations of AgNPs applications. The production of indirubin showed a decrease between about 1.54- and 2.15-fold, compared with the control group. The highest decrease was obtained at the leaf extracts treated with 0.25 mg L^{-1} of AgNPs (about 2.15-fold).

The production of tryptanthrin increased in all the treatments of AgNPs, but the highest increase was observed at a concentration of 2 mg L^{-1} (4.59 ± 0.006 μg g^{-1}), and this increase was 1.71-fold of control (2.68 ± 0.007 μg g^{-1}) on day 5. On 10 and 15 days, although tryptanthrin contents showed a decrease, the concentrations of 2 mg L^{-1} of AgNPs, which had the highest tryptanthrin production, were 1.48- and 1.19-fold, respectively, compared with the control.

Production of indole alkaloids was altered in in vitro cultures of *Isatis* after exogenous application of phytohormones and chemical elicitors. It was reported that while treatments of methyl jasmonate enhanced the production of tryptanthrin and indirubin (indole alkaloids), putrescine treatments decreased the tryptanthrin production and it did not affect the indirubin production in leaf explants of *I. demiriziana* Mısırdalı in vitro grown (Karakas 2019).

It was reported that silver nanoparticles, as chemical elicitors, encouraged the production of important secondary metabolites, synthesized by the medicinal plants (Shahin 2018). The production of atropine showed a significant increase (up to 2.42-fold of control) with treatment of silver nanoparticles in hairy roots of *Datura metel,* depending on treatment times (12, 24 and 48 h) (Shakeran et al. 2015). Jamshidi et al. (2014) reported that taxol production was increased after the treatment with AgNPs to cell suspension cultures of *Corylus avellana* L.

Moreover, many researches showed that silver nanoparticles had also significant effects on the production of phenolic and flavonoid compounds. The production of some phenolic and flavonoids in the in vitro cultures was increased as depending on concentration and exposure time of plants to AgNPs added to MS medium (Jasim et al. 2017; Fazal et al. 2016; Kim et al. 2017; Spinoso-Castillo et al. 2017; Chung et al. 2018). The production of anthocyanin in leaves of plantlets in vitro grown was enhanced after both 1 and

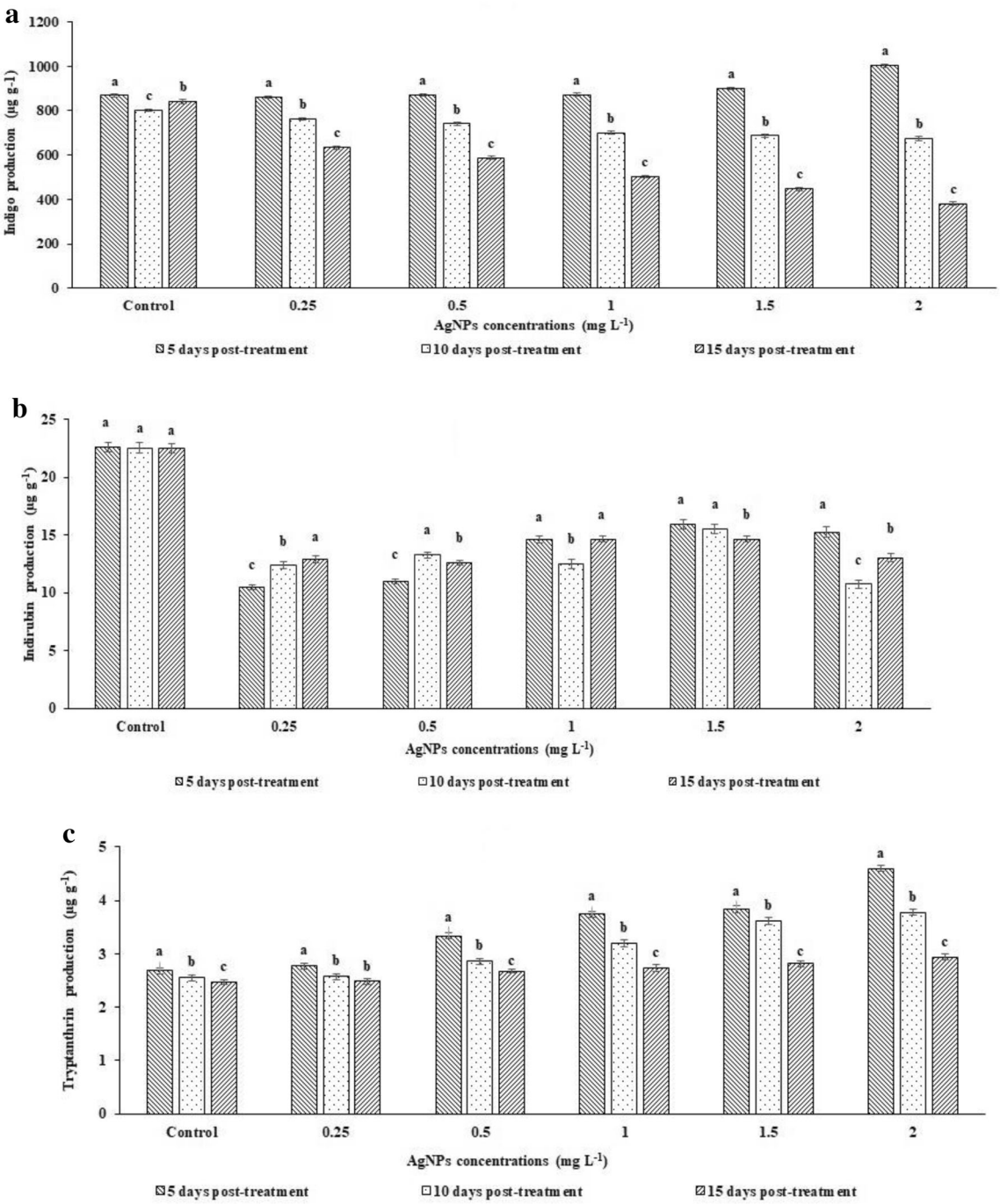

Fig. 4 Effects of AgNPs on the production of **a** indigo, **b** indirubin and **c** tryptanthrin production in the leaves of *I. constricta* plantlets harvested on day 5, 10 and 15 at five concentrations (0.25, 0.5, 1, 1.5 and 2 ppm). Error bars show standard deviations

2 weeks of treatment with 0.5 and 3.0 mg L^{-1} AgNPs, about 5.99-fold that of the control (Qian et al. 2013).

Changes in the production of plant secondary metabolites are associated with oxidative stress, which is caused by the production of excess amount of reactive oxygen species (ROS) after NPs exposure. ROS can also serve as signals for other messengers like jasmonic acid (Wu and Ge 2004), salicylic acid (Baxter et al. 2014), ethylene, etc., which are capable of modulating secondary metabolisms directly or indirectly (Zhang et al. 2016). To avoid the detrimental effects of ROS, a set of antioxidant defense mechanisms are activated in plant cells. Treatment with AgNPs increased the production of phenolics, which might act as antioxidants to scavenge the ROS (Franklin et al. 2009; Comotto et al. 2014. AgNPs triggered ROS signaling and production of anthocyanin and flavonoid in

Arabidopsis thaliana (Sosan et al. 2016). Treatment with AgNPs increased the antioxidant enzymes in *Brassica juncea* (Sharma et al. 2012), *Spirodela polyrhiza* (Thwala et al. 2013) and *Pisum sativum* (Tripathi et al. 2017).

4 Conclusion

The present study demonstrated that the treatments of AgNPs to in vitro grown plantlets showed different effects on indigo, indirubin and tryptanthrin production in leaf explant of *I. constricta* depending on different concentrations and the treatment times. While treatments of AgNPs were not effective for indirubin production for decreasing its quantity, they were effective for indigo and tryptanthrin production for increasing their quantities in in vitro conditions.

Acknowledgements This work was financially supported by the Sirnak University Research Project Council (Grant Number: 2017.04.08.01).

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