



# The physiological and biochemical responses to engineered green graphene/metal nanocomposites in *Stevia rebaudiana*

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## Abstract

*Stevia* is a source of natural sweetener. Numerous researches showed that silver nanoparticles affected the yield and growth of agricultural crops. The effect of silver, graphene, and nanocomposite nanoparticles on a few biochemical and morphological parameters of the *Stevia* plant were studied. Synthesized Ag nanoparticles were confirmed by the absorption maxima and SEM micrograph. The use of nanocomposites at 40 mM, resulted in the higher chlorophyll content (25%) and increased accumulation of soluble sugars (27%), flavonoids (51%), total phenol (33%) and total protein (51%) as compared to the control plants. The plants treated with nanocomposite at a concentration of 60 mM, had more stevioside and rebaudioside content than plants treated with nanocomposite at a concentration of 40 mM.

**Keywords** Graphene · Nanocomposite · Ag nanoparticle · *Stevia rebaudiana* Bertoni · Plant extract

## Abbreviations

AgNP	Silver nanoparticle
BSA	Bovine serum albumin
Chl	Chlorophyll
IAA	Indole-3-acetic acid
G	Graphene
<i>S. rebaudiana</i>	<i>Stevia rebaudiana</i>
CRD	Completely randomized design
LSD	Least significant difference

*Stevia rebaudiana* (*S. rebaudiana*) belongs to the Asteraceae family has a sweetener essence (Lemus-Mondaca et al. 2012). Recently, researchers have used some biotechnology techniques to enhance the plant production for drug production (Jain and Spencer 2006). The high content of sweet steviol glycosides is in *stevia*'s leaves (Geuns 2003). Researchers have focused on the application of nanoparticles in agriculture to improve crop quality,

plant growth and disease control. Graphene is made from a one-atom-thick planar sheet of carbon atoms (Allen et al. 2009). Graphene has ability in environmental remediation application (Miralles et al. 2012). The properties of nanomaterials can be manipulated exactly by controlling the shape, size, and chemical composition of the materials. Nanomaterials can be synthesized with various chemical functional groups, like conjugation with plant products (Mody et al. 2010). To decrease the aggregation reaction of the graphene nanosheets, some inorganic nanoparticles such as metals have been interposed in the interlayer of the graphene nanosheets (Cheng et al. 2011). Metal nanoparticles can be incorporated in the graphene layers to prevent the aggregation of the graphene sheets in the dry state (Muszynski et al. 2008). Plant extracts are a potential source of a bioactive chemical compound that have been confirmed to be able reducing and capping agents for the biosynthesis of a variety of metal nanoparticles (Salunke et al. 2014). Recently, researchers have reported a novel, simple, inexpensive, eco-friendly, and accessible strategy to synthesis graphene with the use of plant extracts (Salunke et al. 2014). *S. rebaudiana* was chosen as the best plant extract to produce stable graphene. Recently, nanomaterial as an inducer is reported to affect the biosynthesis of secondary metabolites in medicinal plant (Gomes et al. 2012). Nanoparticles can increase the reactive oxygen species and hydroxyl radicals' production that deforms the cell membranes, and results in changing of the

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penetrability which facilitates the entry of the nanoparticles into plant cells and then stress induced by the particles results in secondary metabolites production (Kim et al. 2007). In here study, it have evaluated the effect of biologically synthesized silver (Ag) nanoparticle, graphene (G), and nanocomposite Ag/G on growth parameters and secondary metabolites content in *S. rebaudiana*.

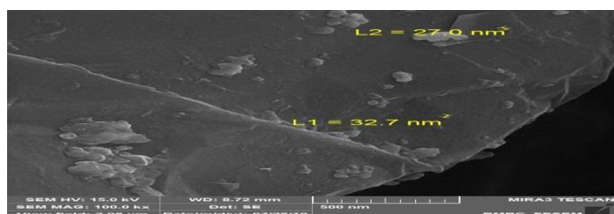
The experiment was done in a randomized design factorial based in a greenhouse. Seedlings of *S. rebaudiana* planted in soil and after the emergence of 4 leaves, treatment (AgNP, graphene and G/AgNP nanocomposite) at different concentration (0, 40, 60 mM) were applied on the plant (2 times in 3 weeks). After three week, the plant was collected for analysis. For the preparation of *S. rebaudiana* leaves extraction, the ethanolic extraction method was used. About 5 g of *S. rebaudiana* leaves were added into a mixture containing 25 ml of ethanolic solvent. The content of the mashed was added into a 250 ml beaker and then mixed again with solvent. The extraction process was performed in darkness (Bujak et al. 2015). For preparation of AgNP, 10 mL of plant extract was added to 90 mL of 1 mmol L<sup>-1</sup> aqueous AgNO<sub>3</sub> solution for the reduction of Ag<sup>+</sup>. The nanoparticles were synthesized by stirring for 2 h at 60 °C. The synthesized nanoparticles were purified by repeated centrifugation at 15 000 rpm for 20 min followed by re-dispersion of the pellet in deionized water. The purified dried powder of nanoparticles was used for different analyses. For synthesis of graphene, the approach was included low power sonication of expanded graphite (Samjung C & G, Korea, 1–10 g/L) in plant extracts (50 g/L) for 24 h by maintaining the temperature lower than 30 °C with a continuous flow of water in the ultra-sonication bath (JACUltrasonic 4020P). After sonication, the dispersion was left to keep overnight to enable the separation of graphite aggregates. The stably dispersed solution of graphene was collected and centrifuged at 1500 rpm for 90 min to get a graphene solution. For preparation of nanocomposites, expanded graphite (1 g) was added to 100 mL of synthesized nanoparticle solution and then sonicated in the ultrasonication bath (JAC-Ultrasonic 4020P) for 1 day at 60 °C. After preparation of treatments, all treatments were sprayed on plant leaves. Pods were in a greenhouse with 16 h of light, and 8 h of darkness. Finally, all treated plants were collected for the assessment. Fresh and dry weight of root and height of shoots were determined at the end of the experiment. UV–visible spectra of the nanocomposites were taken. After freeze-drying of the purified nanocomposites, the structure and composition were analyzed by SEM (Carl Zeiss, LEO-1530). For biochemical assay leaves were used to measure chlorophyll content (Arnon's 1949). For carotenoid content determination, additions of 25 mL of acetone were made to obtain a paste, which was transferred into a funnel (5 µm) coupled

to a 250 mL Buchner flask and filtered under vacuum. The extract obtained was transferred to a 500 mL separatory funnel containing 40 mL of petroleum ether. The acetone was removed through the slow addition of ultrapure water (Milli-Q-Millipore). Then, the extract was transferred through a funnel to a 50 mL volumetric flask containing 15 g of anhydrous sodium sulfate. The volume was made up of petroleum ether, and the samples were read at 450 nm (Arnon's 1949). For total carbohydrate determination, proteins were removed from the ethanolic extracts at 0 °C for 15 min. The extracts were concentrated by evaporating ethanol at 70 °C under vacuum. Extracts were mixed with chloroform and centrifuged for 5 min. Total carbohydrates content was determined using enthrone modified method of Yemm & Willis (1954). For analysis of total protein concentration in plants was assayed by Bradford method (1976). Bovine serum albumin (BSA) was used as the standard protein and the total protein concentration was measured by a spectrophotometer at 550 nm. For determine the flavonoid contents, dried plant materials (10 mg) were extracted with 75 ml (95%) ethanol at 4 °C. The solvent was evaporated at 40 °C under a reduced pressure chamber. The evaporated extract was used for further analysis. To determine the total flavonoid content, the aluminum chloride colorimetric method was performed, and quercetin was used to generate the standard calibration curve. The absorbance was measured at 420 nm using a spectrophotometer. The total flavonoid content was calculated as mg g<sup>-1</sup> FW (Muszynski et al. 2008). For extraction and HPLC analysis of glycosides, 100 mg of dry leaves were mixed in 10 ml of pure methanol for 25 min. Then the methanol was evaporated and added to n-hexane (25 ml) for neutralization. After evaporation of the solvent, 5 mM of solution (including water and acetonitrile was added and filtered to use in HPLC analysis using an Aqua C-18-125A (150 × 4.0 mm, 5 microns) from Phenomenex (Torrance, CA, USA). Extract (10 µl) was injected into a column with specimens of Cosmosil NH<sub>2</sub>-MS with a length of 15 cm, a diameter of 4.6 mm attached to the HPLC device of the Unicam-crystal-200 model. All samples were analyzed under isocratic conditions. The mobile phase consisted of distilled water (20%) and acetonitrile (80%), which passed through the column at a constant flow rate of 1 ml min<sup>-1</sup>. A diode array detector was used at a wavelength of 210 nm. The pump pressure was set at 800 psi and the amount of each substance was compared to the standard courier (Mamta et al. 2010). Glycosides were used as a standard. For statistical Analysis, the experiments were arranged as factorial completely randomized design (CRD). Analysis of variance and significance of differences among means were performed by one-way ANOVA and least significant difference (LSD) methods by SAS software (SAS Institute, Cary, NC).

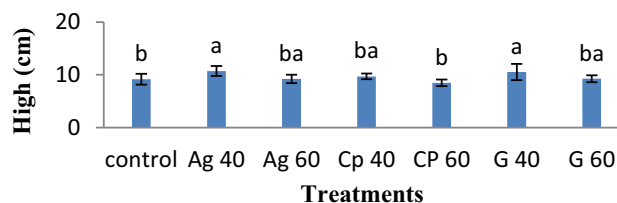
SEM images showed that the synthesized AgNP have average sizes of about 32.7 nm and absorption bands of about 400 nm in UV–vis spectra (Fig. 1).

Result of morphological assay showed that synthesized AgNP and graphene at 40 mM increased plant height (10.73 cm) but high concentrations of nanocomposites (60 mM) had an inhibitory effect on plant height (8.50 cm) (Fig. 2).

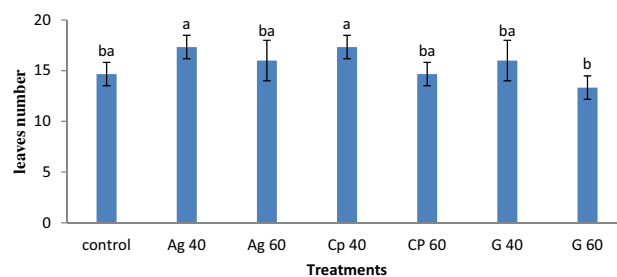
The results also showed that the plant treated with AgNP and nanocomposite with a concentration of 40 mM has more leaf numbers compared to other concentrations (Fig. 3). The results showed that the synthesized AgNP and nanocomposite at 40 mM concentration had the greatest effect on the enhancement of dry and fresh weight (0.44 g). Graphene at 60 mM concentration had the least effect on plant fresh weight (1.53 wt%) (Fig. 4). According to Fig. 5, nanocomposite at concentration of 40 mM had the greatest effect on root length (8.5 cm). Graphene at 60 mM concentration had significant effect on root length in comparison to control treatment (7 cm). Nanocomposite at 40 mM had most positive effect (0.72 mg/gfw) and graphene at 40 and 60 mM had a most negative effect (0.22 mg/gfw) on chlorophyll a content. But no difference was observed between AgNP at concentration of 40 mM and nanocomposites (Fig. 6a). Nanocomposite at a concentrations of 40 and 60 mM had the greatest effect on the amount of chlorophyll b (0.67 mg/g fresh weight), as well as the graphene at concentration of 60 mM (0.55 mg/g fresh weight) showed the lowest effect on chlorophyll b (Fig. 6b). Using different concentrations of AgNP, graphene and nanocomposites on total chlorophyll resulted in biochemical changes in stevia plants. The highest amount of total chlorophyll was observed in plants treated at 40 mM nanocomposite (0.95 mg/gfw). There was no significant difference in the total chlorophyll content of the plant treated with 40 mM nanocomposite and 40 mM AgNP (Fig. 6c). Nanocomposite treatments at 40 and 60 mM and the AgNP at 60 mM had the greatest effect on carotenoid content (1 mg/g fresh weight) (Fig. 7). Result of biochemical assay showed that the highest flavonoids content were produced in the treated plant with nanocomposites at concentrations of 40 and 60 mM (40 mg quercetin per dry weight) (Fig. 8). The results showed that the



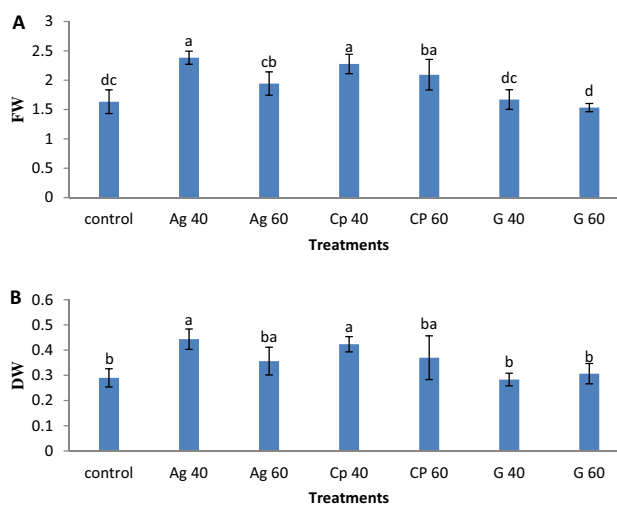
**Fig. 1** SEM image of synthesized Ag/Graphene nanocomposite on the leaves of stevia in different magnification



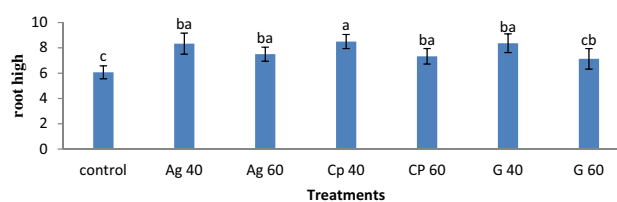
**Fig. 2** Effect of synthesized silver nanoparticles (Ag40 and Ag 60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 and CP60) on height of stevia



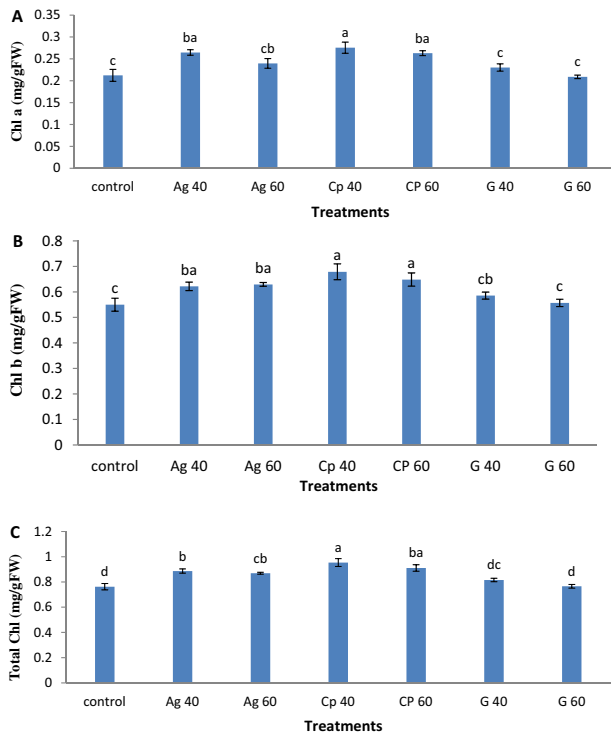
**Fig. 3** Effect of synthesized silver nanoparticles (Ag40 and Ag60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 and CP60) on leaves number of stevia plant



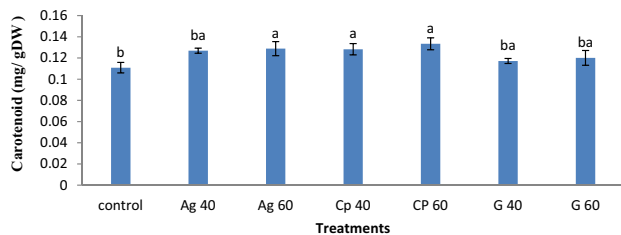
**Fig. 4** Effect of synthesized silver nanoparticles (Ag40 and Ag60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 and CP60) on fresh weigh (a) dry weight (b) of stevia plant



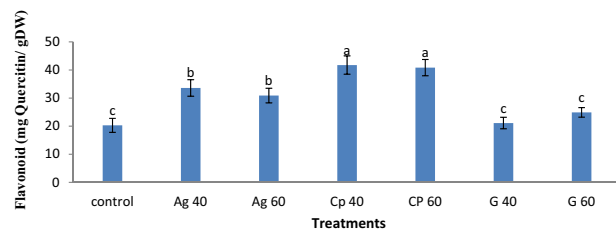
**Fig. 5** Effect of synthesized silver nanoparticles (Ag40 and Ag60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 and CP60) on root height of stevia plant



**Fig. 6** Effect of synthesized silver nanoparticles (Ag40 and Ag60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 ad CP60) on chlorophyll a (a) chlorophyll b (b) and total chlorophyll (c) of stevia plant



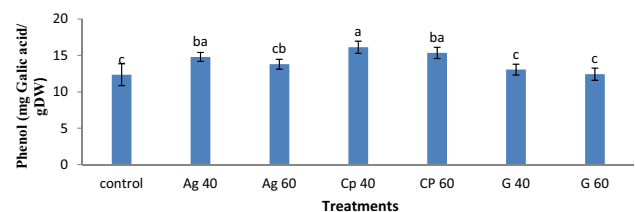
**Fig. 7** Effect of synthesized silver nanoparticles (Ag40 and Ag60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 ad CP60) on carotenoid of stevia plant



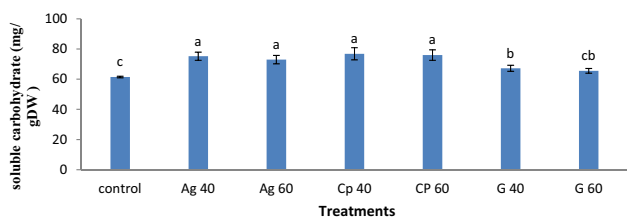
**Fig. 8** Effect of synthesized silver nanoparticles (Ag40 and Ag60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 ad CP60) on flavonoid content of stevia plant

nanocomposite treated plant showed the highest phenol content (40 mg Gallic acid/g dry weight) at 40 mM concentration. Significant differences were also observed in

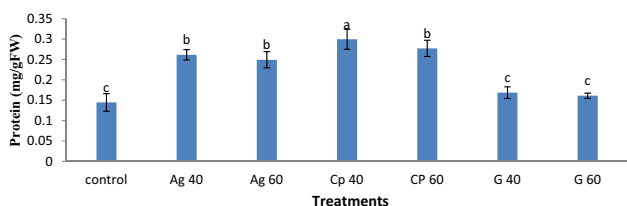
plants treated with 40 mM AgNP and 60 mM nanocomposites (Fig. 9). The results indicated that the highest amount of soluble carbohydrate (76.71 mg/g dry weight) was observed in plant treatments with AgNP and nanocomposites at concentrations of 40 and 60 mM (Fig. 10). The results showed that the plants treated with 40 mM nanocomposite had the highest total protein content, but the plants treated with AgNP at 40 and 60 mM concentrations showed almost similar total protein content (0.26 mg/g dry weight) (Fig. 11). HPLC analysis of *S. rebaudiana* extract showed the presence of two major peaks and four smaller peaks based on authentic standards; two major peaks were identified as stevioside, and 2 were identified as rebaudioside A. Other smaller peaks were identified as: rebaudioside B, C, F and dulbecauside A. The retention time of glycosides was  $5.06 \pm 0.8$  min and  $6 \pm 0.38$  min (Fig. 12). Plants treated with nanocomposite at a concentration of 60 mM have shown an increasing trend over of stevioside content than other treatments. Analysis of results on rebaudioside A showed that plants treated with 60 mM graphene (19 mg/g dry weight) had shown the highest rebaudioside A content in all treated plants. A comparison of the result about Rebaudioside F showed that the different treatments did not have any significant difference in rebaudioside F glycosides. Changes of rebaudioside C content showed that the highest content of this type of glycosides were observed in the nanocomposite treated plants (1.47 mg/g dry weight). Plant treated with nanocomposite at 60 mM showed the highest dulbecauside A content (3.48 mg/g dry weight). Rebaudioside B content in the foliar treatment had an increasing trend compared to the control. The highest content of rebaudioside B was observed in plants treated with 60 mM AgNP and 60 mM graphene (2.14 mg/g dry weight). The overall results of this study showed that plants treated with nanocomposite at 60 mM concentration had shown the highest effect among different plant treatments. Also, a significant increase was observed compared to the control sample (Fig. 13). Nanocomposites treatment was more effective on enhancing glycosides content than AgNP, and graphene alone in stevia plant. Synthesis of AgNP, graphene and nanocomposites showed concentration-



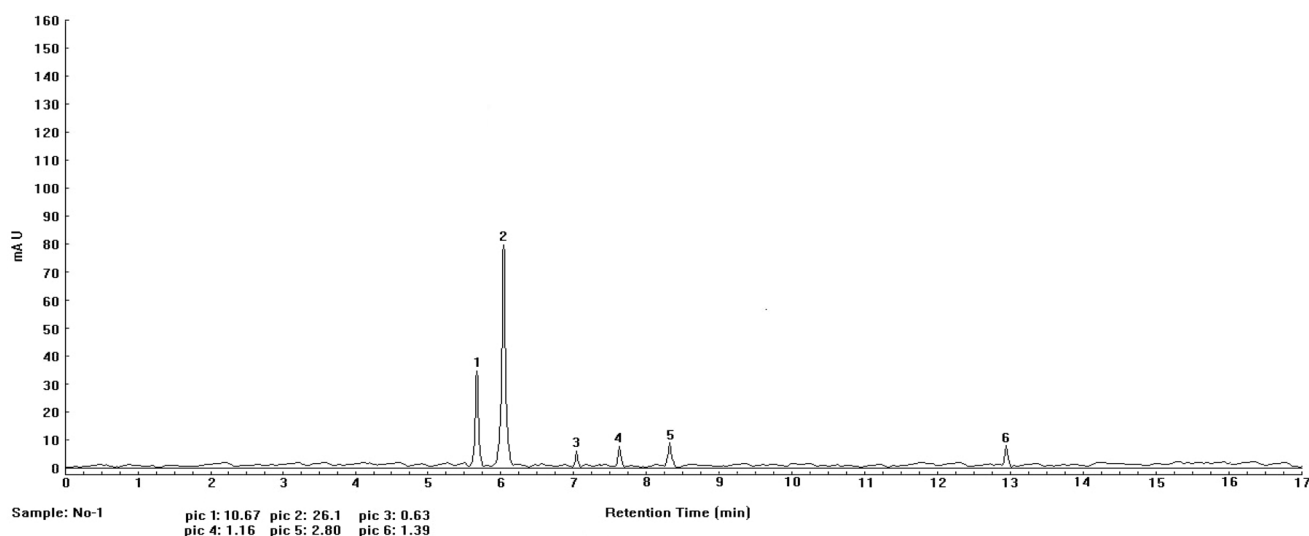
**Fig. 9** Effect of synthesized silver nanoparticles (Ag40 and Ag60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 ad CP60) on phenol of stevia plant



**Fig. 10** Effect of synthesized silver nanoparticles (Ag40 and Ag60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 ad CP60) on total carbohydrate of stevia plant

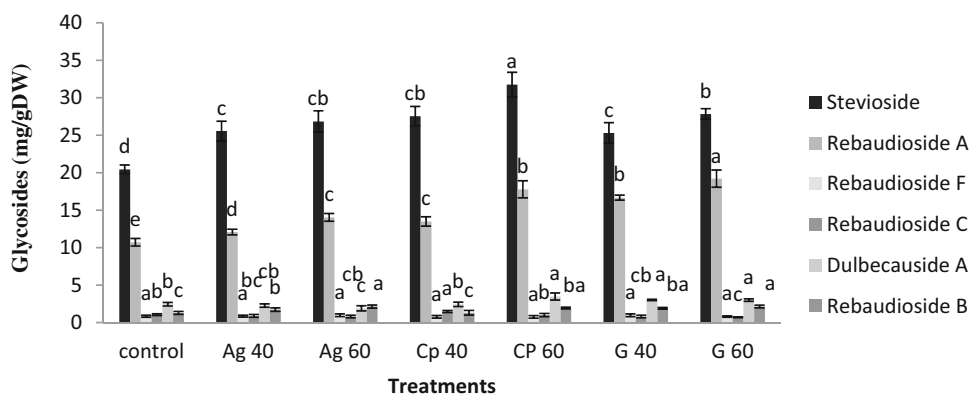


**Fig. 11** Effect of synthesized silver nanoparticles (Ag40 and Ag60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 ad CP60) on protein content of stevia plant



**Fig. 12** chromatograms of on main glycosides of *S. rebaudiana*

**Fig. 13** Results of HPLC analysis on main glycosides content of plant treated with different concentrations of synthesized silver nanoparticles (Ag40 and Ag60), graphene nanoparticles (G40 and G60) and nanocomposites (CP40 ad CP60)



dependent behavior (El-Temseh and Joneer 2012). So that in treated plant with higher concentrations of nanocomposites (60 mM), more glycoside content was observed, especially in stevioid and rebaudioside A content. Stimulation of the absorption spectra of the plasmon of the extracts results in the appearance of brown color at different wavelengths in the range of 300 to 700 nm that in this study was shown at a maximum wavelength of 480 nm (Kumar and Nirmala, 2004). Surface plasmon resonance (SPR) bands depend on the shape, size, composition, morphology and dielectrics around the environment of green synthesized silver nanoparticles (Li et al. 2011). The SEM image showed spherical shape and 32.7 nm in size of synthetic AgNP. It can be concluded that the size and morphology of AgNP are related to the interaction between the reduction of biological supplements such as alkaloids, terpenoids, and flavonoids with metal atoms (Moteriya and Chanda 2014; Ramezani et al. 2017; Majlesi et al. 2018). Also, the concentration of treatments plays an important role in stimulating the biochemical process and biosynthesis of

secondary metabolites in stevia plants. Research has shown that AgNP increase plant growth by blocking the ethylene signaling pathway. In addition, it has been confirmed that silver ions bind to specific receptors in plant cells to inhibit ethylene function (Rezvani et al. 2012). Plant treated with AgNP regulates the expression of genes related to ABA signaling pathway, IAA biosynthesis, etc., followed by increased growth factors (Arase et al. 2012). Increasing in the production of end-products of the phenylpropanoid pathway such as phenol, flavonoids and anthocyanin content in plants under stress is important to the stability properties of elicitors (Daayf et al. 2000). AgNP can regulate key genes in the biosynthetic pathways of anthocyanin and flavonoids in *A. thaliana* (Vecerova et al. 2016). Graphene can affect protein production, that way; different nanoparticles can activate different enzyme pathways. In addition, increased protein content may be due to the energy required for plant mechanisms against unusual changes in stress conditions (Larcher 2010). The potential effects of graphene depend on many factors such as the physical and chemical state, concentration and timing of exposure to the plant, the biological effects of the nanomaterials on its size, chemical composition, solubility, shape, surface structure and aggregation. Small size and high surface area increase the uptake and interaction with biological tissue, resulting in different biological effects in living cells (Nel et al. 2006). Graphene inhibits the biosynthesis of chlorophyll and acts as a layer on the plant's leaf, ultimately reducing plant growth. The main mechanism for phototoxicity of graphene involves in the effects of mechanical damage and shading, which cause darkening and light scattering completely carried out at the plant surface, and consequently, the rate of photosynthesis is reduced (Zhao et al. 2017). The results of this experiment showed that nanocomposites could have a greater impact due to the activity of two nanoparticles, which alone could not perform such a function. The results of this study have shown the best concentration of synthesized NP in the application of agriculture.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that have no conflict of interest.

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