

Investigation on some main glycosides content of *Stevia rebaudiana* B. under different concentrations of commercial and synthesized silver nanoparticles

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ARTICLE INFO

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Article history:

Received: Jan 8, 2018

Accepted: Mar 20, 2018

Keywords:

Silver nanoparticle,
green synthesis silver
nanoparticle,
plant extracts,
Stevia rebaudiana

ABSTRACT

Stevia rebaudiana B. extract as a low-calorie sweetener has gained worldwide approval. Synthesized nanoparticles by plant have been used in different programs such as medical, pharmaceutical and agricultural fields. Here, we report the increase of the main glycosides of stevia plant with silver nanoparticles (AgNPs) treatment. This fact has stimulated research on the analysis and comparison of different concentrations of commercial and synthesized AgNPs on the glycosides of stevia plant by high-performance liquid chromatography (HPLC). After AgNPs synthesis, spectrum absorption of extracts was analyzed in different ranges from 300 to 700 nm and the maximum peak was observed at 420 nm. Transmission electron microscopy (TEM) micrograph of synthesized AgNPs from *Stevia rebaudiana* extract showed spherical shape with the size of 25 nm. A comparison between quantities of glycoside by HPLC under both treatments of AgNP exhibited that synthesized AgNPs were more effective than commercial AgNPs on stevioside and then rebaudioside. On the other hand, results showed that increasing AgNP concentration in both treatments will lead to glycoside content enhancement. Overall, significant changes were observed in the glycosides in plants under synthesized and commercial AgNP treatment that could be used to create plants with higher quality of glycosides for pharmaceutical approaches.

Citation: Pharm Biomed Res 2018;4(1): 8-14. DOI: [10.18502/pbr.v4i1.140](https://doi.org/10.18502/pbr.v4i1.140)

Introduction

Nanomaterials (nanoparticles, NPs) have a range of specific properties such as chemical, physical, and optical properties, which are not found in macromolecules (1, 2). Silver nanoparticles (AgNPs) have strong surface area development, which leads to some especial characteristics and properties like high catalytic activity, reactivity, adsorption ability, and antimicrobial activity (3, 4). These properties can be used in medicine, cosmetics, pharmaceuticals, and industrial detergents. Physical, chemical, and biological (green synthesis) methods are employed for the production of AgNPs (5).

Physical and chemical AgNP synthetic methods have many disadvantages; therefore, the green synthetic method has garnered considerable attention. In the biological synthesis methods of AgNPs, various plant species can be used. Biological synthesis methods do not require the usage of hazardous chemical compounds, high pressure, temperature or energy, do not generate hazardous waste, and do not need purification (2, 6, 7, 8). In the green synthetic methods, the ability of plant extracts as natural reducing agents is

exploited. Several plant extract ingredients may act as natural reducers, such as polyols, amino acids, terpenoids, glycosides, vitamin E, polyphenols, and geraniol. Many researchers reported that the biological synthesis of NPs is due to the presence of natural stabilizers like proteins and polyols, which display greater stability in plant extraction (2, 6, 9).

Stevia rebaudiana (*S. rebaudiana*) belongs to Asteraceae family and grows on high latitudes with long days. *S. rebaudiana* species is considered as a sweetener in all kinds of candies, drinks, and sweets because of its natural dietary sugar. *S. rebaudiana* extract has varied properties like antiviral and antioxidant agents (9). Recently, researchers have used biotechnological techniques to increase secondary metabolites production as renewable sources of drug production because of limited natural resources of medicinal plants. In biotechnological techniques, variance in different plant species can be created by utilization of induced NP and plant breeding (10). For example, Laguta et al. (11) studied the positive effect of green synthesized AgNPs on the flavonoids and hydroxycinnamic acids content with different

extraction methods. According to reports of Varshney *et al.* (12), a plant-derived compound with potential usage in AgNP synthesis is glycosides in *S. rebaudiana*. Glycosides can reduce silver ions and play the role of natural stabilizers in biological synthesis of NPs. According to the research by Huang and Yang (13) polysaccharides are the reducing agent of silver ions, whereas negative charge of heparin is the stabilization agent of NPs. In *S. rebaudiana*, rebaudiosides A, B, C, D, E and dulcoside A are most effective in the reduction of silver ions and forming NPs (14).

According to the high efficiency of NPs by plants in different programs in medical, pharmaceutical and agricultural fields, this study focuses on determination of the best concentration for glycosides induction and clarifies which treatment (biological synthesized silver NPs and commercial silver NPs) is more effective in glycosides content of *Stevia rebaudiana* plant, which can be used for medicinal applications.

Materials and Methods

This factorial experiment was based on a completely randomized design in a research greenhouse of Sana Institute of Higher Education. Seedlings of *S. rebaudiana* were purchased after planting and assuring of successful establishment and the emergence of 4 to 6 leaves of the plant, treatment with green synthesized silver nanoparticles (prepared using ethanolic extract and silver nitrate according to the following method in different concentrations of 0, 10, 20, and 40 mM) was applied on the plants by spray solution (twice during three weeks). This experiment was performed with three replications in each treatment (green synthesized AgNP and commercial AgNP).

Preparation of extracts

Ethanol extraction method was used for the preparation of *S. rebaudiana* leaf extract. The solvent was a mixture of water and ethyl alcohol and ethyl alcohol alone (in a 1:1 ratio). About 5 g of *S. rebaudiana* leaves was added to a mixture containing 25 ml of ethanolic solvent and pounded for 15 min. Then, the content was added to a 250 ml beaker and then mixed again with addition of 80 ml of the solvent. The extraction process was performed in darkness by a mechanical stirrer with a rotational speed of 750 rpm during 3 h. The extract was filtered twice through Whatmann filter paper No 1 and then stored in tubes in the dark at 4°C (15). Pods were transported to a greenhouse with 16 hours of light (8000 to 10000 Lux light) and 8 hours of darkness, relative humidity of 60% and average temperature of $5 \pm 25^\circ\text{C}$. The nutritional requirements of plants were provided by a Hoagland nutrition solution.

Synthesis and monitoring of AgNPs

For AgNPs synthesis, 50 ml of silver nitrate (as a standard 0.001 M solution) was added into an Erlens-

meyer flask. About 0.5 ml of *S. rebaudiana* extract was transferred and mixed at room temperature (25°C) for 24 h by a magnetic stirrer (250 rpm) in the light. After 0, 15, 30, and 45 min of reduction, synthesis of the AgNPs (Ag ions converted to AgNPs) was monitored using UV-VIS spectroscopy.

Silver nanoparticles characterization

Specification of green synthetic AgNPs was performed by transmission electron microscopy (TEM). The morphological analysis (size and shape) of the particles was performed by TEM. Aqueous AgNO₃ sample was loaded on a grid of carbon-coated copper, and the solvent was evaporated at room temperature for an hour. The TEM micrograph picture was recorded on Zeiss - EM10C instrument on carbon coated copper grids with an accelerating voltage of 80 kV. The microscopic picture was recorded and documented in different magnification ranges.

Extraction and high-performance liquid chromatography (HPLC) analysis of stevioside and rebaudioside A

100 mg of dry leaves was mixed in 10 ml of pure methanol for 25 minutes. Then, the methanol was evaporated at 48°C and added to n-hexane (25 ml) for neutralization. After evaporation of the solvent, 5 ml of a solution including water and acetonitrile (20: 80) was added and filtered to be used in HPLC analysis using an Aqua C-18-125A (150 × 4.0 mm, 5 micron) from Phenomenex (Torrance, CA, USA). Afterwards, 10 µl of the extract was injected into chromatography column with specimens of Cosmosil NH₂-MS with a length of 15 cm, a diameter of 4.6 mm, and a diameter of 5 micrometers attached to the HPLC device (Unicam-crystal-200). The mobile phase consisted of distilled water and acetonitrile with isocratic conditions, which passed through the column at a ratio of 80% of acetonitrile and 20% water at a rate of 1 ml/min. 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 by comparing the inhibition time of the output courier and the surface area under the curve (all the solvents were purchased from Sigma-Aldrich company) (16).

Statistical Analysis

The experiments were performed in a completely randomized design (CRD). Analysis of variance and significance of differences among means were evaluated using one-way ANOVA and least significant difference (LSD) methods by SAS software (SAS Institute, Cary, NC) and the graphs were drawn using Microsoft Office Excel 2010.

Results

Color change of mixture after biological synthesis nanoparticles production

After nanoparticles production, the color of the mixture was converted to dark brown. Reduction of AgNO_3 to AgNPs during treatment of the *S. rebaudiana* extract was evident from the color change of the reaction mixture from yellow to brown by exposure to sunlight (Figure 1). The excitation of the surface plasmon vibrations led to brown color of the mixtures.



Figure 1 Color change from green (before adding NaNO_3) to brown (after adding NaNO_3 and synthesized Ag nanoparticles formation), from ethanolic extract of *S. rebaudiana*

The UV-vis spectra recorded from the stevia reaction from different times and OD values are plotted in Figure 2. Absorption spectrum of *S. rebaudiana* extract at different wavelengths ranging from 300 to 700 nm revealed a peak at 435 nm (Figure 2).

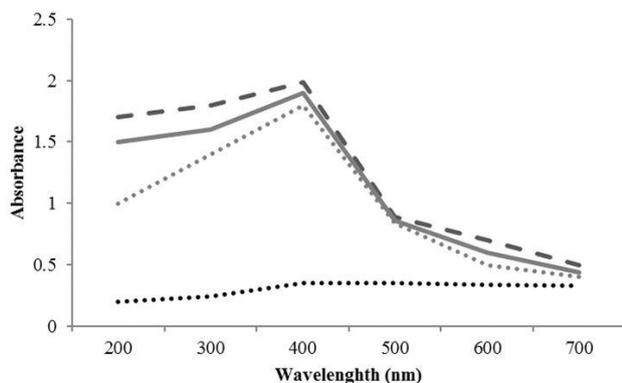


Figure 2 UV-visible spectrum of biosynthesized AgNPs showed peak at 435 nm after time course (0, 15, 30, and 45 minutes).

400 nm in the UV-vis spectra (16, 17). This shows that the green synthetic AgNPs were spherical, which has been confirmed by TEM. TEM analysis revealed that the obtained AgNPs were spherical in shape with an average size of around 25 nm (Figure 3).

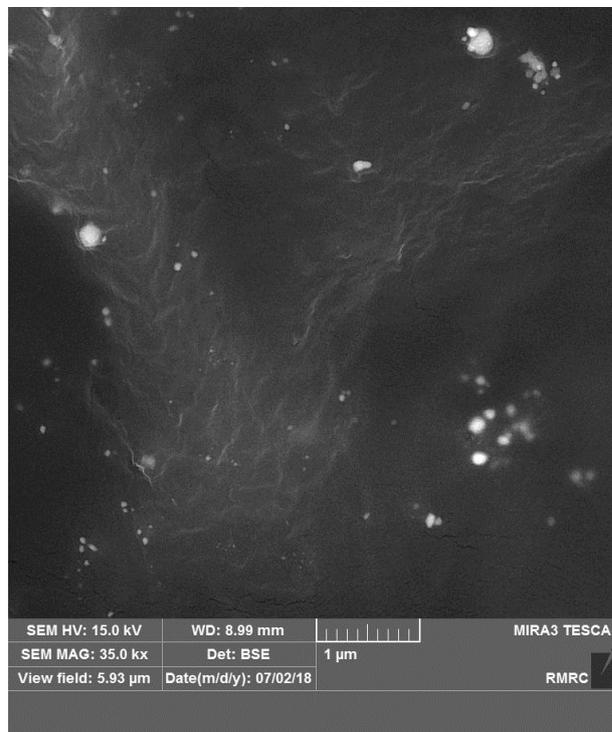


Figure 3 TEM micrograph of synthesized AgNPs from ethanolic extract of *S. rebaudiana*.

HPLC analysis of stevioside and rebaudioside A content
HPLC analysis of *S. rebaudiana* extract indicated the presence of two major peaks based on authentic standards; one was identified as stevioside and the other was identified as rebaudioside A. Other smaller peaks were identified as: rebaudioside B, C, F, and dulcoside A. The mean retention times of stevioside and rebaudioside A were 5.05 ± 0.8 min and 6 ± 0.2 min (Figure 4). The overall results of this study revealed that the treatment of plant with green synthetic and commercial AgNPs in different concentrations led to change in glycosides content of stevia plants with a notable increase in stevioside and rebaudioside A content than control. As shown in figures 4 and 5, a significant difference was observed between treatments compared to control plants ($P < 0.05$). Figure 5 reveals glycosides content ranging from 0.5 to 38 mg/g dry.w under different concentrations of commercial AgNP treatments. According to Figure 5, glycosides content ranged from 0.5 to 42 mg/g dry.w under different concentrations of biological synthesis AgNP treatment. The results showed that the highest amount of stevioside (42-38 mg/g dry.w) and rebaudioside A (15-13 mg/g dry.w) were assigned to

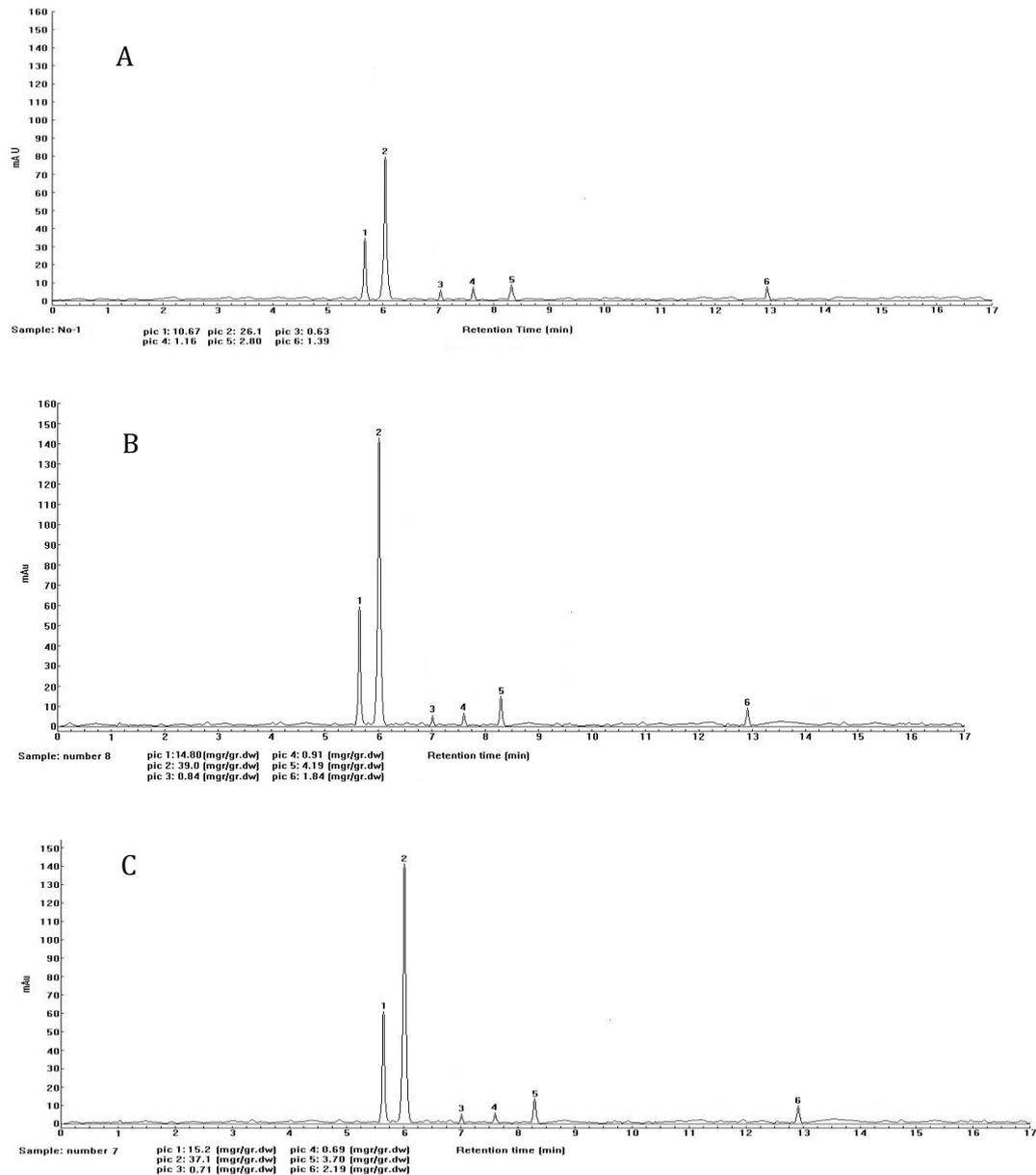


Figure 4 Chromatograms of concomitant treatments at A: control B: 20 mM concentration of commercial AgNP C: 20 mM concentration of synthesized AgNP on main glycosides of *S. rebaudiana*

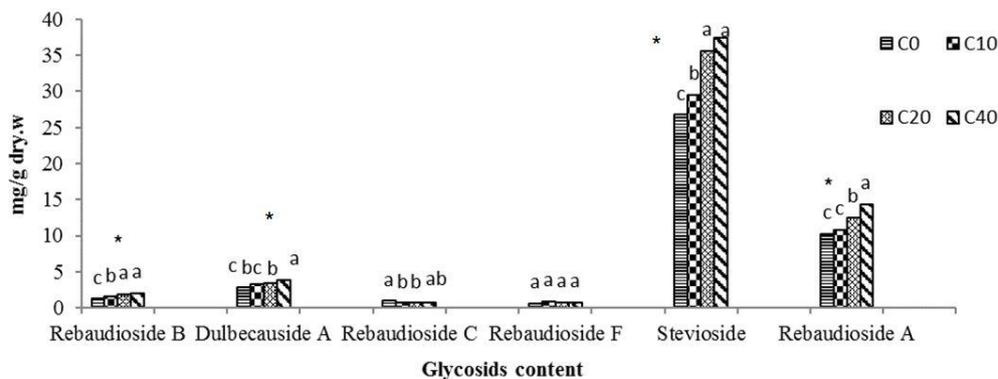


Figure 5 Effect of commercial AgNP applications in (in different concentrations of C0, C5, C10, C20, C40 mM) on main glycosides (mg/g dry.w) level in leaves leaf tissues of *S. rebaudiana* plants. The mean values were obtained from three independent experiments. *Significantly ($P \leq 0.05$) different according to LSD: least significant difference; data represent the mean \pm SD, n=3. In each column, means that have at least one letter in common did not show any significance differences.

samples treated with the green synthesis and commercial AgNP agents at the 40 mM concentration and the lowest content of all glycosides was exhibited in 10 mM concentration of both AgNP treatments. The glycosides content appeared with gradual increase along with the increasing of AgNP concentration in all biological and commercial treatment cases, with some exceptions in rebaudiosid A content in 20 and 40 mM concentrations in green synthesis AgNP (Figure 6). A comparison between different treatments of green synthesis and commercial AgNP on all glycosides content exhibited that green synthesis AgNP treatment in different concentrations was more effective (20%) than commercial AgNP treatment.

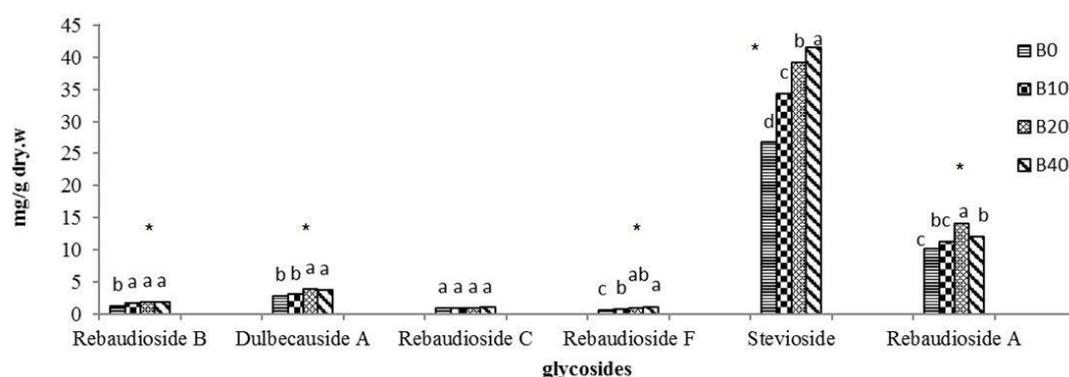


Figure 6 Effect of green synthesis AgNP applications in (in different concentrations of B0, B5, B10, B20, and B40 mM) on main glycosides (mg/g dry.w) level in leaves leaf tissues of *S. rebaudiana* plants. The mean values were obtained from three independent experiments. *Significantly ($P \leq 0.05$) different according to LSD: least significant difference; Data represent the mean \pm SD, $n=3$. In each column, means that have at least one letter in common did not show any significance significant difference.

Discussion

In this study, we described green synthesis of AgNPs using *S. rebaudiana* leaf extracts. The influence of green synthesis of AgNPs on the glycosides content of *S. rebaudiana* was investigated. Also, the shape and size of NPs and their absorbance were measured.

Elicitors positively affect the production of secondary metabolites (19). Recently, some researchers reported that nanomaterials influence on the production of pharmaceutical and commercial secondary metabolites in medicinal plants (20).

In this study, the effect of commercial and synthesized AgNPs on stevia was evaluated. Laguta *et al.* (11) studied the effect of green synthesized AgNPs on the flavonoids and hydroxycinnamic acids content. Their results showed enhancement of green synthesized AgNP-treated plants. So far, no studies have compared the effects of commercial and synthesized AgNPs in different concentrations on main glycosides of stevia plant.

Nanoparticles have a positive effect on the pharmacological properties of medicinal plants, such that NPs act on target sites associated with a physiological

process through synergistic actions with several biological compounds (21). Some changes mediated by NPs in secondary metabolism could also be beneficial if used in a physiological process that NPs as elicitors can enhance the biosynthesis of desired secondary metabolites. Artemisinin (22) and diosgenin contents (1) as important drugs were increased in plants treated with NPs. Purification of specific biological compounds can be performed using green synthetic NPs as they are detected to conjugate with secondary metabolites (23).

Altogether, results of this research demonstrated positive elicitation effect of AgNPs on the cellular pathways involved in the production of stevioside and rebaudioside A content in *S. rebaudiana* and

concentration of elicitor acts as an important role on stimulation of physiological process and secondary metabolite biosynthesis in *S. rebaudiana*.

According to the results of this study, treatment of *S. rebaudiana* by synthesized and commercial AgNPs leads to an increase in glycosides, especially stevioside and rebaudiosids A.

Some researchers have reported that NP treatment of plants resulted in the enhancement of phenolic production (24, 25, 26). So far, many studies have reported that NPs impact the secondary metabolism of plants. For example, secretion of phenolic compounds on an extracellular medium after treatment with TiO_2 NPs was increased up to 22% in *Arthrospira platensis* and *Haematococcus pluvialis* (24). A substantial increase in artemisinin content was observed (39%) after AgNP treatment in *Artemisia annua* L. hairy root cultures (22). This increase was related to oxidative stress (H_2O_2 production), CAT activity, and lipid peroxidation. Plant growth and diosgenin concentration were increased in *Trigonella foenum-graecum* L. exposed to AgNP treatment (26). Isovitexin and ferulic acid were

enhanced in barley after NP treatment (1). AgNPs can up-regulate key genes of anthocyanin and flavonoid biosynthesis pathway in *A. thaliana* (27).

Although all the reports mentioned above showed evidence for modulation of plant secondary metabolism mediated by NP, several enzymes and secondary metabolites have enhanced the metallic nanoparticles formation from the relative ionic compounds. The reduction reaction mainly involved natural products such as organic compounds, proteins, pigments, polysaccharides, and plant resins. Plant secondary metabolites contribute to the reduction reaction to synthesize green NPs.

Although there is a large body of evidence indicating that NPs can modulate natural products through interfering with different signaling pathways, the accurate mechanism of this regulation is not fully understood. According to some studies, it is believed that the early responses of plants to NP treatment are enhanced in reactive oxygen species (ROS) levels, cytoplasmic Ca^{2+} efflux, and up-regulation of mitogen-activated protein kinase (MAPK) cascades because of the following finding. AgNPs were recognized by plasma membrane-bound receptors, then they stimulate Ca^{2+} burst and ROS induction in *A. thaliana* (28, 29). Ca^{2+} levels and some proteins related to signaling pathways are up-regulated in AgNP treated with *O. sativa* root extract (30). Researchers believe that AgNPs, or released ions, by binding to Ca^{2+} channels, Ca^{2+} receptors, and Ca^{2+}/Na^+ ATPases activity, block cell metabolism. NP-specific proteins or calcium-binding proteins, NPs imitate Ca^{2+} in the cytosol (31). MAPK phosphorylation followed by down regulation of transcription factors activation leads to changes in the transcription of secondary metabolite in plants (32-35).

A comparison between commercial and green synthesis of AgNPs showed that leaf extract treatment of green synthesized AgNPs was more effective in increasing the glycosides of *S. rebaudiana* than commercial AgNPs. A similar result was observed comparing function of commercial and green synthesis AgNPs on cell lines (36). According to the results of this research, AgNP in two treatments (synthesized and commercial) showed a concentration-dependent behavior, such that in higher concentrations (40 mM) of AgNPs more glycoside content, especially stevioside and rebaudioside A, was observed.

Solubility of NPs and ROS production affects activity of NPs (37). As a result, NPs enter plant cells easier resulting in secondary metabolites production (38). El-Temsah and Joner (39) reported that AgNPs in different concentrations and sizes affect seed germination differently.

Moteriya and Chanda (40) reported that the reducing potential enhanced by increasing concentration of AgNPs. Kanipandian et al. (41) also showed similar results in green synthesized AgNPs of *Cleistanthus collinus* extract.

Synthetic AgNPs formation by the reduction of $AgNO_3$ during treatment of the *S. rebaudiana* extracts is evident from yellow to brown color change of the reaction mixture by exposure to sunlight (Figure 1). Study of Moteriya et al. (42) exhibited similar color change of extract of *Cassia roxburghii* that demonstrates formation of AgNP particles.

Excitation of the surface plasmon vibrations absorption spectrum of extracts leads to appearance of the brown color at different wavelengths ranging from 300 to 700 nm, and in this study a maximum peak at wavelength of 435 nm was observed (43). The surface plasmon resonance bands depend on shape, size, composition, morphology and dielectric around the environment of the green synthesized AgNPs (44). The TEM micrograph showed spherical green synthesized AgNPs with a size of 25 nm. According to Figure 3, we can conclude that the size and morphology of AgNPs is related to the interactions between metal atoms and reducing biomacromolecules like alkaloids, terpenoids, and flavonoids (45, 42).

Conclusion

This study proposed that synthetic AgNPs treatment is an expensive approach to produce specific medicinal compounds from medicinal plants, such as stevioside and rebaudioside A used for diabetes.

Acknowledgements

We would like to thank Sana Institute, Sari, Iran, for cooperating in plant growth.

Funding

This paper was supported by Sana Institute, Iran.

Authors' contributions

MR designed the experiment, ZM performed the experiment, MG and MR analyzed the data, MR, MG and ZM interpreted the data and performed the final revision of the manuscript. MR, MG and ZM approved the final revision of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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