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Original Article

Photocatalytic Activity of Titanium Dioxide Nanoparticles (TiO_2) on the Physiological and Phytochemical Properties of Stevia [*Stevia rebaudiana* (Bertoni) Bertoni]

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Abstract

This study investigated the photocatalytic effect of titanium dioxide nanoparticles on some physiological and phytochemical properties of stevia plant. Stevia plant was treated with eight concentrations of titanium dioxide nanoparticles (0, 20, 40, 60, 80, 100, 200 and 400 µL/L). After 3 weeks of plants treatment, samples were collected for analysis of chlorophyll, PAL enzyme activity, total phenol and flavonoid content. Also extraction of plant for assessment of steviosides and rebaudiosides glycosides by HPLC was performed. The results showed that treatment of titanium dioxide nanoparticles at 400 µL/Lconcentration had the highest and at control had the lowest effect on chlorophyll content of leaves, total phenol, total flavonoid and Phenylalanine ammonia lyase enzyme activity. Glycoside content showed that treatment of nanoparticle at 200 µL/L concentration had the highest and control concentration had lowest effect on stevioside content. Rebaudiosides content showed that, nanoparticle at 400 µL/L had highest effect and at 20 and 40 µL/L had lowest effect on rebaudioside A and B content. But, nanoparticle at 80 µL/L had maximum effect, and at 20 and 40 µL/L had lowest effect on rebaudioside C and F content. It was concluded that varied concentration of nanoparticles has different effect on glycosides content that this results could apply for further technologies in agriculture industry.

Keywords: Stevia, TiO_2 nanoparticle, Photocatalytic, Photochemistry

Introduction

Stevia plant [*Stevia rebaudiana* (Bertoni) Bertoni], known as honey leaf, belonging to the Asteraceae family [1,2]. This plant is important for diabetic patients, because its sugars have a very low absorption [3]. The leaves of this plant contain vitamins C and A [4]. Important characteristics of stevia in agriculture can be perennial and cold resistance, economical production of stevia leaf [5]. Nanotechnology, with the help of new tools, is capable of food absorb in plant. In agricultural industry, it can also be used intelligent conductor system and sensors to fight viruses and pathogens in agricultural products [6]. Also, nanotechnology can work in the context of intelligent toxins. These poisons contain a molecular code that allows them to move into the plant and into the area where the pest or plant is attacked, then released and killed by the pest [7].

Titanium dioxide nanoparticles (TiO_2) are capable of improving light absorption and rubisco enzyme activity [8], increasing nitrate uptake [9] and accelerating the conversion of inorganic matter [10] to increase plant dry and fresh weight. The positive effect of TiO_2 nanoparticles on photosystem II and thylakoid membrane has also been reported [11]. Using of TiO_2 nanoparticles can stimulate cell division, increase cell size and also stimulate callus formation under dark conditions and may have similar effects to plant hormones (cytokinin and gibberellin [12]. It was also found that TiO_2 nanoparticles increased light absorption, accelerated the transfer and

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conversion of luminous energy, prevented the deterioration of chloroplasts and increased the photosynthetic period of chloroplasts [11], so it transfers luminous energy to electrons and converts them to chemical energy and ultimately increases CO_2 stabilization [13].

An important property of TiO_2 nanoparticles that makes it very useful in life is its photocatalytic properties [14].

TiO_2 nanomaterials, are capable by absorbing UV radiation through their photocatalytic activity make antibacterial coating, in addition to preventing the radiation from passing through the material.

The TiO_2 nanoparticles are coated on the surface of suitable substrates such as glass or silica compounds and placed in ponds exposed to ultraviolet light [14].

The most commonly used TiO_2 photocatalyst is the photonic decomposition of organic compounds. TiO_2 is used as a photocatalyst in the disinfection of various environmental contaminants such as organic matter, viruses, bacteria, fungi, algae and cancer cells [15]. In this case, the substance converts to harmless CO_2, water and inorganic anions. This efficiency is attributed to the high oxidation of hydroxyl (OH^0) and radicals, which are known as strong oxidizing agents [16].

Therefore, this study aimed to investigate the photocatalytic effect of TiO_2 nanoparticles on physiological and phytochemical properties of Stevia plant under controlled conditions.

Material and Methods

Plant Materials

This study was performed in 2016 in the laboratory of Islamic Azad University, Damghan Branch in a completely randomized design with three replications.

Stevia plant species were prepared and transferred to pots (20 cm diameter and 22 cm height) containing perlite and soil (50: 50 ratio) for 10 days to grow and root in the growth chamber that was set at 25°C under 18 h photoperiod and 6 h dark.

Nanoparticle Characters

TiO_2 nanoparticles were used as suspension of TiO_2 formulation in the US-Nano company. Crystalline white nanoparticles were 20 nm in diameter, molecular weight of 79.93%, purity of 99.9%, specific surface area of 200 m^2/g and a combination of anatase and rutile (80% anatase and 20% rutile) (Fig. 1).

Treatment levels

Plant samples were treated with suspension of TiO_2 nanoparticles at different levels (0, 20, 40, 60, 80, 100, 200 and 400 µL/L). So that after 11^{th} day of the establishment (after appearance of first leave), TiO_2 nanoparticles treatments sprayed on the leaves of all plants, and the next stage of spraying was one week later and the last stage took two weeks.

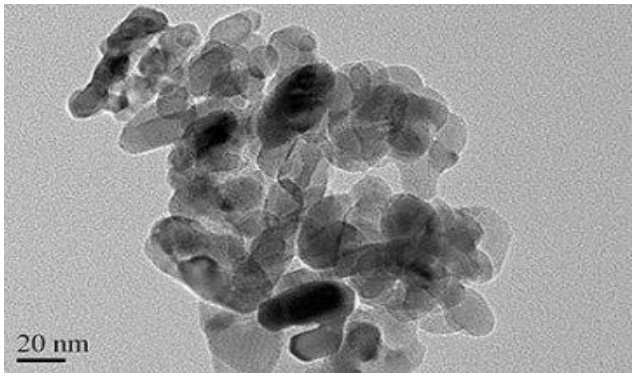

Fig. 1 Scanning electron microscope (SEM) image of TiO_2 nanoparticles.

Assessment of Physiological Characters:

Photosynthetic pigments: Chlorophyll (a, b and total chlorophyll)

Fully developed leaves were used to measure the amount of chlorophyll [17]. The formulas bellow was used to find the dosage of chlorophyll a and b. Here are Arnon's [17] equations:

Chl a = $15.65\ A_{666}$-$7.340\ A_{653}$

Chl b = $27.05\ A_{653}$-$11.21\ A_{666}$

Total Chl x+c = $(1000\ A_{470}$-$2.860\ C_a$-$129.2\ C_b / 245$

Phytochemical Characters

Assessment of Total Flavonoid Content

The aluminum chloride calorimetric method was utilized to determine the total flavonoid content. The dried plant materials (10 mg) were extracted using 75 mL of methanol (95%) and aluminum chloride (10% methanol), 0.1 mL of potassium acetate (1M) and 2.8 mL of distilled water at 4°C. The quercetin was applied to create the standard calibration curve. To prepare the quercetin solution, quercetin (5.0 mg) was dissolved in methanol (1.0 mL). Then, standard quercetin solution (0.6 mL) was mixed with 2% aluminum chloride (0.6 mL). The mixture was incubated for 60 min at room temperature. The absorbance was analyzed at 415 nm by spectrophotometer. The total flavonoid content was considered as mg/g FW [18].

Assessment of Total Phenol Content

The amount of total phenolic compounds was determined by Folin-ciocalte reagent. Each of the plant methanol extracts (0.5 mL of 1:10 mL/g) separately with 5 ml of diluted folin. The sample was diluted (1:10 diluted with distilled water) and then 4 mL of sodium carbonate solution (Na2co3) was added.

The samples were kept at laboratory temperature for 15 minutes and read at 765 nm with a spectrophotometer [19].

PAL Activity Analysis

Preparation of enzyme extract: for preparation of crude enzyme extract, 0.3 g leaf tissue was homogenized in 15 mL of 0.05 M phosphate buffer (phosphate buffer, 6.5 mM (PH=7) 0.5 mM EDTA) with pH 8.8. The homogenate was centrifuged at 5,000 g for 15 min. The supernatant was used for further assay. The extraction was carried out at 4 °C. 0.1 mL enzyme extraction was mixed with 0.3 mL L-phenylalanine (50 mM). Phosphate buffer (0.05 M) was added to the reaction mixture (3 mL). After incubation of the reaction mixture at 30 °C for 15 min, the absorbance was recorded at 290 nm using a spectrophotometer (Biochrom WPA Biowave II). For determination of product, the standard curve of cinnamic acid was drawn. PAL activity was calculated as a unit/mg Protein (U/mg Protein) [20].

Measurement of Glycosides Characters by HPLC

Extraction of Steviosides and Rebaudiosides A, B, C and F

For this purpose, 100 mg dry leaf was added into 10 mL of pure methanol and mixed for 15 minutes. It was then evaporated in the refrigerant at 45 °C with methanol and the residue was evaporated by adding 20 mL of n-hexane and then after evaporation of the solvent 5 mL of solution (including acetonitrile and water (80:20) was added, dissolved and filtered to be injected into the HPLC [21]. Extract was prepared for injection in HPLC using an Aqua C-18–125A (150 × 4.0 mm, 5 micron) from Phenomenex (Torrance, CA, USA). Amount of 10 µl of the extract was injected into chromatography column with specimens of Cosmosil NH_2-MS with a length of 15 cm, a diameter of 4.6 mm and a diameter of 5 micrometers attached to the HPLC device of the Unicam-crystal-200 model. The mobile phase consisted of distilled water and acetonitrile with isocratic conditions, which passed through the column with 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 standard courier by comparing the inhibition time of the output courier and the surface area under their curve (all solvent was purchase from Sigma-Aldrich Company).

Statistical Analysis

After technical and biological experiments, all data was analyzed based on a factorial randomized design (CRD) and was done in three biological repeats for each treatment. Variance between various means and its impact was analyzed using one-way ANOVA and Duncan (P<0.05) methods in SPSS software (version 23, SPSS, Chicago, IL). The mean comparison was executed using SAS software (SAS Institute, Cary, NC), followed by drawing graphs with MS Office excel.

Results and Discussion

The results of comparison mean of data showed that the effect of TiO_2 nanoparticles treatment on total chlorophyll, total phenol and flavonoid content, phenylalanine ammonium enzyme (PAL) and glycosides of stevioside, ribaudioside A, B and F were significant in stevia plant.

Effect of foliar application of TiO_2 nanoparticles on photosynthetic pigments: chlorophyll leaves (a, b and total)

Comparison mean of leaves chlorophyll content (a, b and total) showed that with increasing different levels of TiO_2 nanoparticles, leaf chlorophyll content increased. So that the concentration of 400 µL/L TiO_2 nanoparticles had the highest (53%) and control samples the lowest chlorophyll a, b and total chlorophyll content of leaves (Fig 2, 3 and 4).

Titanium promotes growth by increasing chlorophyll and photosynthesis, especially by increasing electron transfer from 2 to 1 photosynthesis, photosynthetic optical activity, and uptake of elements involved in chlorophyll production and photosynthesis such as iron, magnesium, and nitrogen [22]. Since TiO_2 nanoparticles increase nitrogen uptake and metabolism, so the rate of chlorophyll synthesis in the plant increases [23].

Mishra *et al*. [24] in a study on tomato plants showed that high concentrations of TiO_2 nanoparticles (200 µg/l) increased plant chlorophyll content to 1.97 mg/g fresh weight.

In another study, investigating the effect of different concentrations of TiO_2 nanoparticles on rice plant showed that lower concentrations of TiO_2 nanoparticles treatment on plant increased chlorophyll a, chlorophyll b and total chlorophyll that can be attributed the presence of more cells per leaf weight [25].

A study by Hashemi and Dehkordi [26] investigated the effect of TiO_2 dioxide nanoparticles (anatase) on the physiological properties of the berry quince (*Fragaria ananassa* c.v. Queen Elisa). These researchers found that nanoparticleat the highest concentration (11.5 mg/l) increased all studied characters of plant (leaf chlorophyll content, soluble solids to titrable acid ratio, vitamin C content, fruit formation percentage, fresh and dry weight of root and plant yield).

Researchers believe that titanium has an effect on the biochemical activities of the plant and increases the activity of catalase, nitrate reductase and peroxidase.

The reason for the increased activity of these enzymes under the influence of titanium treatment was due to increased iron uptake.

Growth of pepper plant was due to the increase in plant length. Titanium increased photosynthesis by increasing nitrogen uptake and consequently increased pepper plant length. It has also been reported, that after titanium treatment, quality of pepper and its sugar content increase due to chlorophyll production [27].

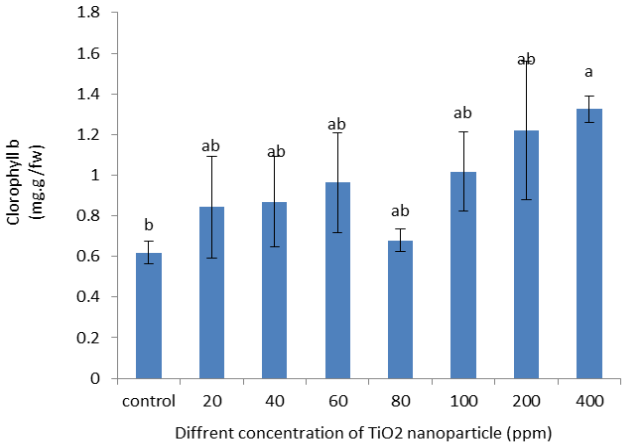

Fig. 2 Effect of TiO_2 nanoparticles on chlorophyll a content in stevia plant

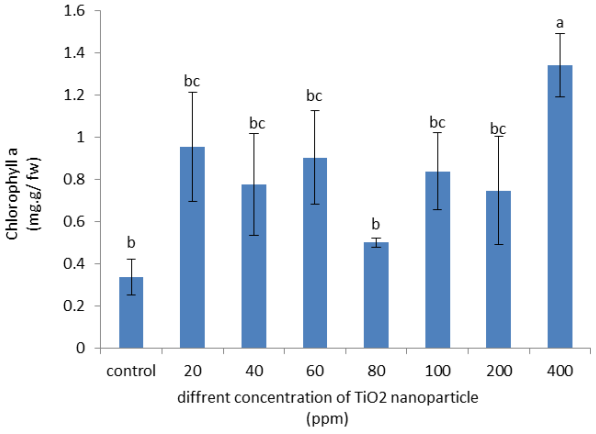

Fig. 3 Effect of TiO_2 nanoparticles on chlorophyll b content in stevia plant

Effect of TiO_2 nanoparticles on total phenol and flavonoid content

Comparison mean values of total phenol and flavonoid showed that by increasing levels of TiO_2 nanoparticles treatment, the activity of these biochemical parameters increased. Treatment of TiO_2 nanoparticles at concentration of 400 µL/L has the highest effect on total phenol (51%), and flavonoid (40%) content compare to control samples (Fig. 5, 6).

Kamalizadeh *et al.* [28] investigated the effect of TiO_2 nanoparticles on the content of rosmarinic acid and chlorogenic acid production in the *Dracocephalum moldavica* L., which results showed that production of two component increases at low concentration of nanoparticles (30 µg/l) and decreased at higher concentrations.

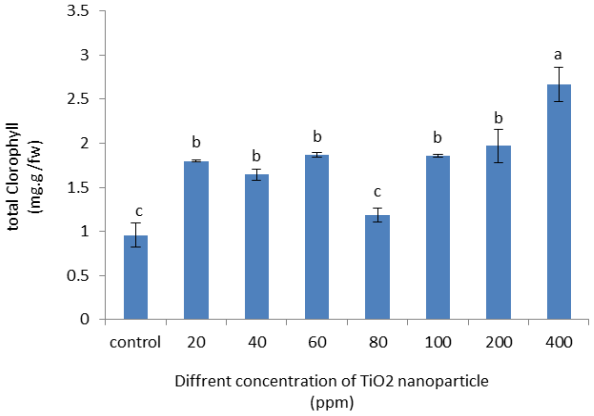

Fig. 4 Effect of TiO_2 nanoparticles on total chlorophyll content in stevia plant

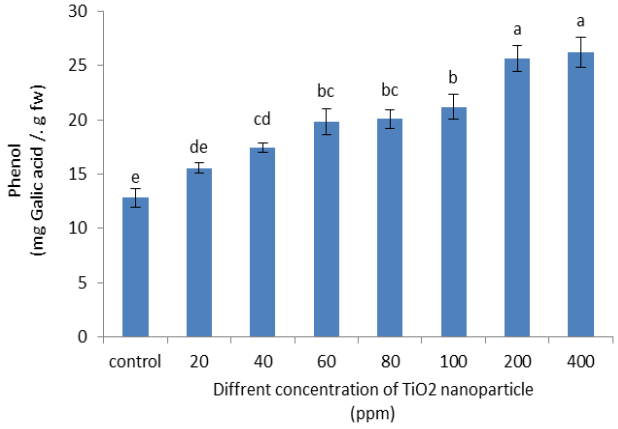

Fig. 5 Effect of TiO_2 nanoparticles on total phenol content in stevia plant

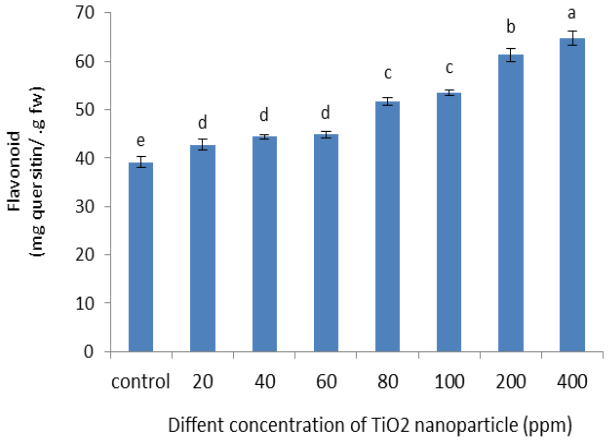

Fig. 6 Effect of TiO_2 nanoparticles on flavonoid content in stevia plant

But Bagal *et al*. [29] conducted an experiment on the effect of TiO_2 nanoparticles on the phenolic and flavonoid content of *Pinus taeda* callus. According to the results, the maximum amount of phenol and flavonoid was observed in the samples treated with high concentration (250 µg/l) of TiO_2 nanoparticles and with decreasing of these nanoparticles concentration the end-products of phenylpropanoid pathway decreased [29].

Effect of TiO_2 nanoparticles on the phenylalanine ammonium enzyme activity (PAL)

Comparison of the average activity of phenylalanine ammonium enzyme (PAL) showed that by increasing levels of TiO_2 nanoparticles, the activity of this enzyme was improved. So that TiO_2 nanoparticles treatment at 400 µL/L has highest effect on PAL activity (73%) than control samples (Fig. 7).

According to the results of a research in *Pinus taeda*, changes in PAL activity, phenolic and flavonoid content were affected by different ratios of TiO_2 nanoparticles and were positively correlated, which it indicated the direct role of PAL on the induction of phenylpropanoid pathway.

Maximum total phenolic and flavonoid content were observed in the samples treated with high concentration (250 µL/L) TiO_2 nanoparticles. By decreasing nanoparticles concentration, the phenylpropanoid content decreased. Positive correlation of PAL enzyme activity and accumulation of phenolic compounds indicates a key role of this enzyme in the biosynthesis of phenolic compounds in *Pinus taeda*. According to the results, it is concluded that PAL as the first and most important enzyme involved in the production of polyphenolic compounds was affected by the TiO_2 nanoparticles treatments [29].

Jordan *et al*. [30] in their research showed that TiO_2 nanoparticles enter the leaf tissue and may cause toxicity in plants, while enzymatic activity (including enzymes Phenylalanine ammonia lyase and its biomarkers) increase, ultimately counteracts with the adverse effects of ROS.

Effect of TiO_2 nanoparticles on stvioside glycosides and rebaudiosides A, B, C and F

Comparison of the mean values of glycosides (stevioside and rebaudiosides A, B, C and F) showed that with increasing different levels of TiO_2 nanoparticles, the amount of these glycosides increased.

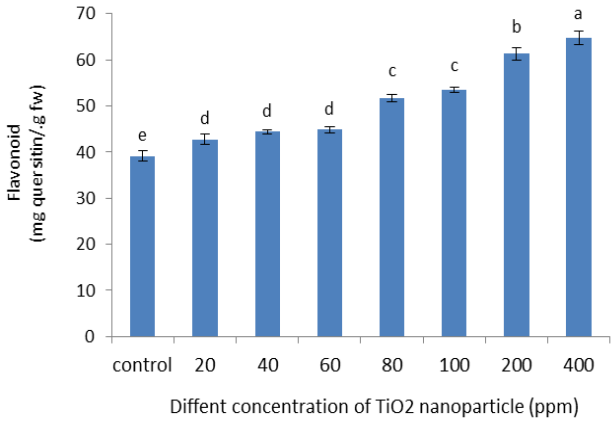

Fig. 7 Effect of TiO_2 nanoparticles on PAL enzyme activity in stevia plant

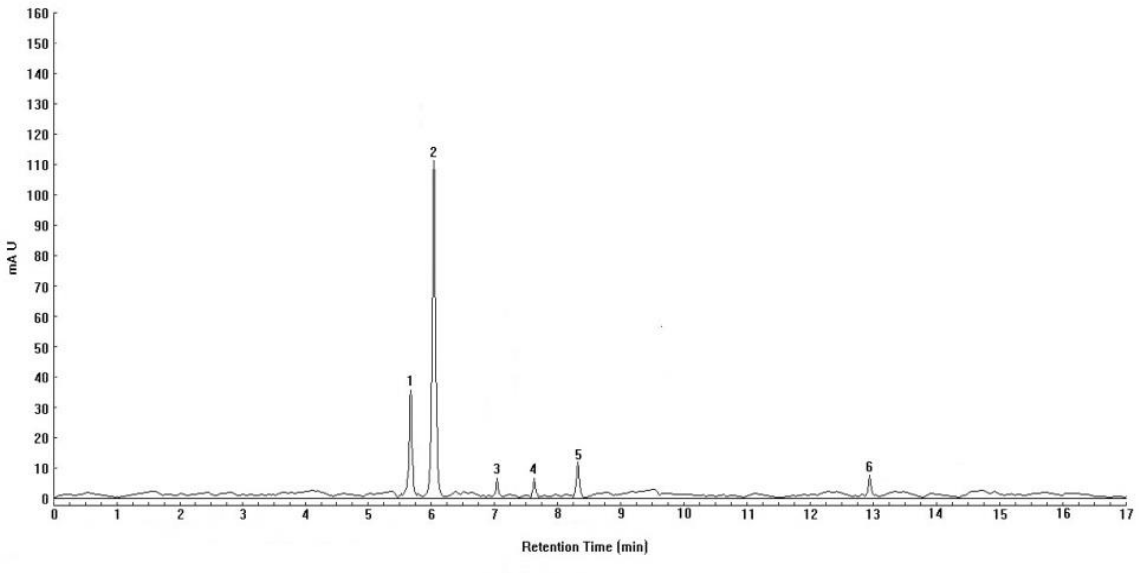

Fig. 8 Chromatogram of HPLC in stevia extract sample. Numbers in horizontal axis showed retention time of different glycosides appeared in stevia plant extraction. Sharp peaks showed retention time of main glycosides appeared in HPLC analysis of stevia extraction (rebaudioside A (retention time 5±3), stevioside (retention time 6) rebaudiosides B (retention time 7±3), rebaudiosides C (retention time 8± 2) and rebaudiosides F (retention time 13)).

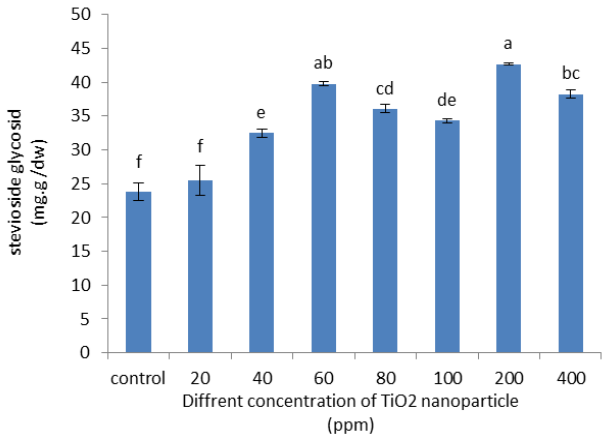

Fig. 9 Effect of TiO_2 nanoparticles on glycoside stevioside content in stevia plant

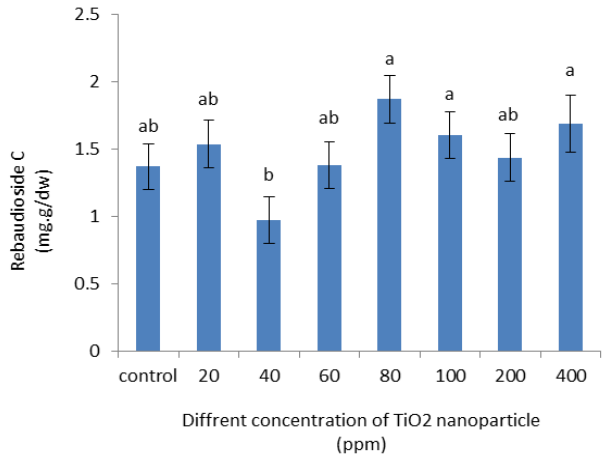

Fig. 12 Effect of TiO_2 nanoparticles on rebaudioside C content in stevia plant

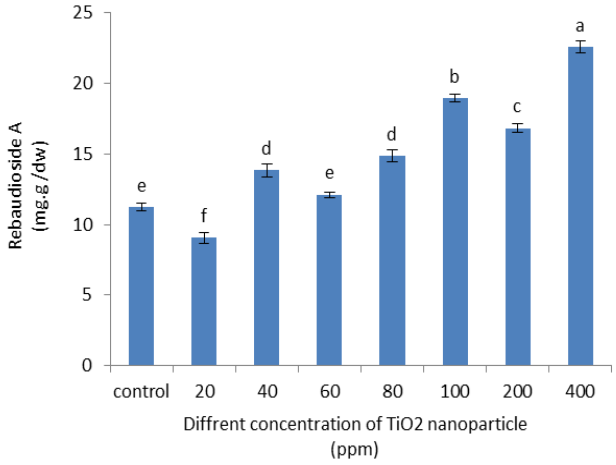

Fig. 10 Effect of TiO_2 nanoparticles on rebaudioside A content in stevia plant

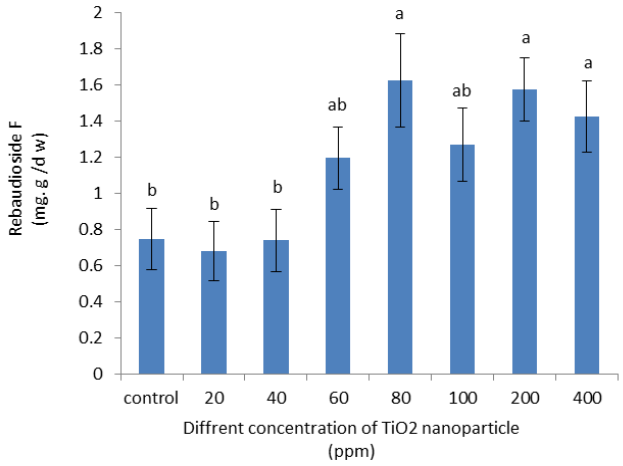

Fig. 13 Effect of TiO_2 nanoparticles on rebaudioside F content in stevia plant

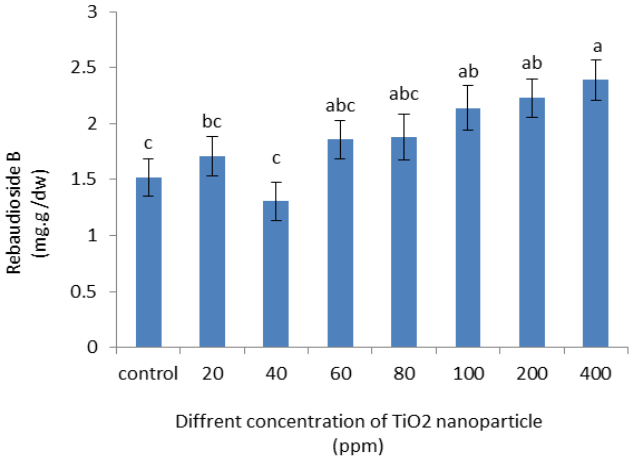

Fig. 11 Effect of TiO_2 nanoparticles on rebaudioside B content in stevia plant

As TiO_2 nanoparticles at concentration of 200 µL/L has highest effect on stevioside glycoside (45%), and at 400 µL/L has highest effect on glycoside rebaudiosides A (50%) and B (34%), and at 80 µL/L, has highest effect on glycoside rebaudiosides C (35%) and F (56%), and also TiO_2 nanoparticles at control sample and at concentrations of 20 and 40 µL/L has lowest effect on glycosides content, respectively (Figure 8, 9, 10, 11, 12 and 13). Stevia leaves contain sweet compounds (steviol glycosides) and steviosides are the main sweet component in stevia that is about 300 times sweeter than sugar and also is a carboxylic alcohol with three molecules of glucose [31,32]. In addition to its interesting sweet properties, it also exhibits many medicinal properties [33-35].

Previous research has shown that the stevioside content present in stevia leaf depends on the cultivar and its growth conditions, and according to studies it can be about 4-20% of leaf dry materials [37].

To our knowledge, no studies on the use of TiO_2 nanoparticles on stevia plant have been reported so far.

However, the results of different study using iron, copper, and silicon nanoparticles on stevia showed that at the higher concentration of iron and copper nanoparticles, the stevioside callus rate (8 µg/l compared to control) increased. And also at the low concentration of the nanoparticles, the amount of stevioside decreased (0.25, 0.5, 1, 2, and 4 µg/l).

But the results of silicon nanoparticles showed that the highest amount of stevioside was observed at low concentrations (0.25, 0.5, 1 and 2 µg/l) and the lowest amount of stevioside was reported at concentrations of 4 and 8 µg/l [37].

Javed *et al*. [38] Reported that the highest levels of steviol glycoside (stevioside and rebaudioside A) were observed in stevia plant in the presence of 1 µg/l concentration of zinc oxide nanoparticles and decreased in high concentrations of nanoparticle (10, 100 and 1000 µg/l).

Also, another research was reported that copper oxide nanoparticles treatment in stevia showed the highest levels of stevioside and rebaudioside A at concentrations of 10 µg/l of copper oxide nanoparticles and at the higher concentrations of nanoparticle (100 and 1000 µg/l), glycoside content was decreased [39].

In a research was investigated on some main glycosides content of *S. rebaudian* under different concentration of commercial and synthesized silver nanoparticles, result showed synthesized silver nanoparticle in low concentration has positive effect on glycoside content and growth factor than chemical nanoparticle in low concentration [40].

Ramezani *et al* [41] in a research investigated effect of silver nanoparticles on expression of some key genes involved in glycosides synthesis pathway in stevia plant. Results of their research showed that plants treated with silver nanoparticles at concentration of 10 and 20 µg/l showed a lower gene expression than the control plant, but plant treated with silver nanoparticles at 40 µg/l concentration showed higher expression genes than plant treated with low concentration of silver nanoparticles and control samples.

It can concluded that some nanoparticle properties such as size, shape, plants species, treatment duration, and condition are important factors in positivity and negativity (toxicity) of the effects silver nanoparticles have on the plant's physiological and morphological aspects. On the other hand, nanoparticles solubility and ROS production affect their activity. Thus, nanoparticles enter plant cells more easily which results in the production of secondary metabolites [42].

Here, the results showed that biosynthesized AgNP treated plants possess increased flavonoids. It is known that the plant's defensive ability depends greatly on the results of the phenylpropanoid pathway products such as flavonoid content which plays a major role in elicitor resistivity [43]. AgNP, can up-regulate main anthocyanin genes and flavonoid biosynthesis pathway in *A.thaliana* [44]. A study on maize by Suriyaprabha *et al*. [45] demonstrated how silver nanoparticles significantly affect phenol and flavonoid content. Lipid peroxidation, CAT activity and oxidative stress (H_2O_2 production) are the causes of this increase.

Govorov and Carmeli [46] reported that chemical energy generation in photosynthetic systems is induced by metal nanoparticles. A novel hybrid system forms after AgNPs bind to the chlorophyll in photosynthetic reaction center due to the increased excitation of electrons induced by plasmon resonance and major acceleration of electron-hole separation.

Application of nanoparticles increases the gas exchange and chlorophyll fluorescence and also affects other factors such as electron transfer rate, stomatal uptake, transpiration, photosynthesis rate (PSII), phytochemical and effective phytochemical productivity [47]. Investigating TiO_2 nanoparticles applications shows improvements in the photosynthesis rate, water conductivity, and transpiration in plant [48]. Treatment of plant with nanoparticles exhibited high photosynthesis rate which resulted in increased nutrients and carbohydrate content due to photosynthesis and also larger shoot and heavier fresh and dry weight [49].

Conclusion

In this research, it was concluded that nanoscale metals showed dissimilar performances than bulk metals. Chemical composition and the physical attributes of nanoparticles are very important in their ability to affect plants. Titanium nanoparticles induce stress since after treating plants with biosynthesized TiO_2 nanoparticles, an increase in PAL activity was recorded. In the present study, biosynthesized TiO_2 nanoparticles demonstrated increased production in glycosides and total phenol and flavonoids content. Thus, by tagging nanoparticles with secondary metabolites, stevia offers opportunities in the nanomedicine field. The results of this study have shown best concentration of synthesized nanoparticle in the application of agriculture and pharmaceutical. It can be used biotechnological processes for production of pure bioactive compounds from plant products.

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References

1. Humphrey TV, Richman AS, Menassa R, Jim E. Spatial organization of four enzymes from *S. rebaudiana* Bertoni that are involved in steviol glycoside synthesis. Plant Mol Biol. 2006;61:47-62.
2. Karuppusamy S. A review on trends in production of secondary metabolites from higher plants by invitro tissue. Organ and cell cultures. J Med Plants. 2009;3:1222-1239.
3. Abdullah R, Alizah Z, Wee WH, Leaw CL, Yeap CB. Immature embryo: A useful tool for oil palm (*Elaeis guineensis* Jacq.) genetic transformation studies. Electron J Biotechnol. 2005;74:25-34.
4. Rita MH. Stevia Nature's Sweeteners. Woodland Publishing Inc, (Web article. 1997:1-29.
5. Ramesh K, Singh V, Megeji NW. Cultivation of *S. rebaudiana* (BERT). Bertoni comprehensive review. Adv Agron. 2006;89:137-177.
6. Nanotechnology-Specialist-Headquarters. Complementary document future strategy (Ten Years Strategy of Nanotechnology Development in the Islamic Republic of Iran) .http://www.nano.ir 5. 2005.
7. Johnson, A. 2005. Agriculture and nanotechnology. Available in: http://www.tahan.com/chalie/nanosociety/course201.
8. Mingyu S, Hong F, Liu C, Wu X, Liu X, Chen L. Effects of nano-anatase TiO_2 on absorption, distribution of light and photo reduction activities of chloroplast membrane of spinach. Biol. Trace Elem. Res. 2007;118:120-130.
9. Yang F, Hong F, You W, Liu C, Gao F, Wu C, Yang P. Influence of nano-anatase TiO2 on the nitrogen metabolism of growing spinach. Biol. Trace Elem Res. 2006;110:179-190.
10. Nair R, Varghese SH, Nair BG, Maekawa T, Yoshida Y, Sakthi Kumar D. Nano particulate material delivery to plants, Plant Sci. 2010;179:154-163.
11. Hong F, Zhou J, Liu C, Yang F, Wu C, Zheng L, Yang P. Effects of Nano-TiO_2 on photochemical reaction of chloroplasts of Spinach. Biol. Trace Elem. Res. 2005;105:269-279.
12. Mandeh M, Omidi M, Rahaie M. In vitro influences of TiO_2 nanoparticles on barley (*Hordeum vulgare* L.) tissue culture. Biol Trace Elem Res. 2012;150:376-80.
13. Gao F, Chao L, Zheng L, Mingyu S, Xiao W, Yang F, Cheng W, Ping Y. Mechanism of nano anatase TiO_2 on promoting photosynthetic carbon reaction of spinach. Biol Trace Elem. Res. 2006;111:239-245.
14. Ding Z, Lu GQ, Green field PF. Role of crystallite phase of TiO_2 in heterogeneous photocatalysis for phenol oxidation in water. J. Phys. Chem. B. 2000;104:4815-4820.
15. Duffy EF, Touati FA, Kehoe SC. A novel TiO_2-assisted solar photocatalytic batch process disinfection reactor for the treatment of biological and chemical contaminants in domestic drinking water in development countries. Sol Energy. 2004;77:649-655.
16. Frazer L. Titanium dioxide environmental knight? Environmental Health. Environ Health Perspect. 2001; 109: A174-A177.
17. Arnon DI. Copper enzymes in isolated chloroplast polyphenol oxide in *beta vulgaris*. Plant Physiol. 1949;24:1-5.
18. Chang CC, Yang MH, Wen HM, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal. 2002;10:178-182.
19. McDonald S, Prenzler PD, Autolovich M, Robards K. Radical scavenging, antioxidant and antioxidant activity of olive extracts. Food Chem. 2001;73:73-84.
20. Vaughn KC & DUKE SO. Function of polyphenoloxidase in higher plants. Physiol Planta. 1984;60:106-112.
21. Mamta PR, Vijaylata P, Arvind G, Bikram S, Ravinde KB Rupinder T, Stimulatory effect of phosphate-solubilizing bacteria on plant growth. Stevioside and rebaudioside-A contents of *S. rebaudiana* Bertoni. Appl Soil Ecol. 2010;46:222-229
22. Owolade O, Ogunleti DO, Adenekan M. Titanium dioxide affects diseases, development and yield of edible cowpea. J Environ Agric Food Chem. 2008;7:2942-2947.
23. Yang F, Liu C, Gao FQ, Su MY, Wu X, Zheng L, Hong FS, Yang P. The improvement of spinach growth by nano-anatase TiO_2 treatment is related to nitrogen photoreduction. Biol Trace Elem Res. 2007;119:77-88.
24. Mishra P, Singh R, Kumar U, Prakash V. *S. rebaudiana* A magical sweetener. GJBBR. 2010;5:62-74.
25. Nonami H, Wu Y, Matthewse MA. Decreased growth-induced water potential a primary cause of growth inhibition at low water potentials. Plant physiol. 2010;114:501-509.
26. Hashemi-Dehkordi A, Mousavi M, Moalemi N, Ghafarian-Moghareb MH. Effect of Titanium Dioxide (Anatase) Nanoparticles on Physiological Characteristics of Strawberry in *Fragaria ananassa* c.v.Queen Elisa under Hydroponic Cultures. Plant Proc Func. 2016;5:79-71.
27. Martínez-Sánchez FM, Nunez A, Amoros J, Gimenez CF. Effect of titanium leaf spray treatments on ascorbic acid levels of *Capsicum annuum* L. fruits. J. Plant Nutr. 2010;16:975-981.
28. Kamalizadeh M, Bihamta MR, Peghambari SA, Hadian J. Effect of different levels of Titanium Dioxide (TiO_2) nanoparticles on two important phenolic compounds in *Dracocephalum moldavica* L. J Res Iranian Herb Flow. 2015;31:435-428.
29. Bagal UR, Leebens Mack JH, Walter Lorenz W, Dean JFD. The phenylalanine ammonia lyase (PAL) gene family shows a gymnosperm specific line age. BMC Genoms. 2016;13:1471-2164.
30. Jordan BR, Strid A, Wargent JJ. Effect of titanium dioxide nanoparticles on hydrolytic and antioxidant enzymes during seed germination in onion. Int J Curr Microbiol App Sci. 2016;3:749-760.
31. Kohda H, Kasai R, Yamasaki K, Murakami K, Tanaka O. New sweet diterpene glucosides from *S. rebaudiana*. Phytochem. 1976;15:981-983.
32. Shibata H, Sonoke S, Ochiai H, Nishihashi H, Yamada M. Glucosylation of steviol and steviol glucosides in extracts from *S. rebaudiana* Bertoni. Plant Physiol. 1991;95:152-156.
33. Gregersen S, Jeppesen PB, Holst JJ, Hermansen K. Antihyperglycemi effects of stevioside in type 2 diabetic subjects. Metab. 2004;53:73-106.
34. Din MSU, Chowdhury MS, Khan MMH, Din MBU, Ahmed R, Baten MA. In vitro propagation of *S. rebaudiana* Bertoin Bangladesh. Afr. J. Biotechol .2006;5:1238-1240.
35. Ferri LA, Alves-Do-Prado W, Yamada SS, Gazola S, Batista MR, Bazotte RB. Investigation of the antihypertensive effect of oral crude stevioside in patients with mild essential hypertension. Phytother. Res. 2006;20:732-736.
36. Geuns JMC. Molecules of interest Stevioside. Phytochem. 2003:64:913-921.

37. Hendawey MH, El-Fadl RA, El-Din TS. Biochemical Role of Some Nanoparticles in the Production of Active Constituents in *S. Rebaudiana* L. Callus. Life Sci J. 2015:12.
38. Javed R, Usman M, Yucesan B, Zia M, Gurel E. Effect of zinc oxide (ZnO) nanoparticles on physiology and steviol glycosides production in micropropagated shoots of *S. rebaudiana* Bertoni. Plant Physiol Biochem 2016:1-6.
39. Javed R, Mohamed A, Yucesan B, Gurel E, Kausar R, Zia M. CuO nanoparticles significantly influence in vitro culture, steviol glycosides and antioxidant activities of *S. rebaudiana* Bertoni. PCTOC. 2017:1-10.
40. Majlesi Z, Ramezani M, Gerami M. Investigation on some main glycosides content of *Stevia rebaudian* B. under different concentration of commercial and synthesized silver nanoparticles. PBR. 2018;4:1-10.
41. Ramezani M, Asghari S, Gerami M, Ramezani F, Abdolmaleki MK. Effect of Silver Nanoparticle Treatment on the Expression of Key Genes Involved in Glycosides Biosynthetic Pathway in *S. rebaudiana* B. Plant Sugar Tech. 2019;22:518-527
42. Navarro E, Baun A, Behra R. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicol. 2008;17:372-386.
43. Govorov AO, Carmeli I. Hybrid structures composed of photosynthetic system and metal nanoparticles: plasmon enhancement effect. Nano Lett. 2007;7:620-625
44. Garcia-Sanchez S, Bernales I, and Cristobal S. Early response to nanoparticles in the Arabidopsis transcriptome compromises plant defence and root-hair development through salicylic acid signaling. BMC Genomics. 2015;16:341-349.
45. Suriyaprabha R, Karunakaran G, Yuvakkumar R, Rajendran V, Kannan N. Silica nanoparticles for increased silica availability in maize (Zeamays L) seeds under hydroponic conditions. Curr Nanosci. 2005;8:902-908.
46. Govorov AO, Carmeli I. Hybrid structures composed of photosynthetic system and metal nanoparticles: plasmon enhancement effect. Nano Lett. 2007;7:620-625.
47. Siddiqui MH, Al-Whaibi MH, Faisal M, Al Sahli AA. Nano-silicon dioxide mitigates the adverse effects of salt stress on *Cucurbita pepo* L, Environ. Toxicol. Chem. 2014;33:2429-2437.
48. Qi M, Liu Y, Li T. Nano-TiO_2 improves the photosynthesis of tomato leaves under mild heat stress. Biol Trace Elem Res. 2013;156:323-328.
49. Kalteh M, Alipour Z, Ashraf S, Aliabadi MM, Nosratabadi AF. Effect of silica nanoparticles on basil (*Ocimum basilicum*) under salinity stress. J. Chem. Health Risks. 2014;4:49-55.