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Investigating the Physiological Responses and the Expression of Effective Genes in Steviol Glycosides Production in Stevia (*Stevia rebaudiana*)

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ABSTRACT

Stevia plant is one of the most important medicinal plants used to control diabetes due to its sweetening properties and low calories. Stevia is cultivated in many parts of the world, and to increase its sweetening properties, the effects of many different factors have been tested on this plant. In this research, we investigated the effect of elements related to metal oxidants on the induction of molecular levels and transcription. Thus, the activity of 3 key genes named *CPPS*, *HDS*, and *GGDPS* in response to six different metal oxidants named Cro_3, Pbo, Fe_2O_3, Ag_2O, Bao, and Tio2 was carried out in this research. The results showed that the increased concentration of metal oxides, especially Fe_2O_3 and TiO_2, escalates gene expression in the biosynthesis of sweeteners extracted from stevia leaves. Also, related to all treatments, the higher the concentration, the higher the gene expression. Among all metal oxide treatments, Pbo and Bao resulted in low gene expression for *CPPS*, *HDS*, and *GGDPS* genes. On the other side, control showed the lowest expression regarding all three surveyed genes, indicating that using metal oxides can achieve higher production of sweeteners in stevia plants. The results of this research determined that physiological characteristics are affected by metal oxide treatments. Also, the expression of genes effective in the production of steviol glycosides, which is one of the important sweetening factors of this plant in the leaves, increases under the influence of these treatments. As a result, it can be said that the use of these treatments can have an increasing effect on the amount of sweetening of the plant.

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1. Introduction

In recent years, the production of natural sweeteners with non-calorific, non-arcogenic and generally healthy is increasing. One of the most encouraging families of diterpenoid glycosides is named steviol glycosides. They are usually found in the leaves of *Stevia rebaudiana* Bertoni (Mirzaei and Shakoory-Moghadam, 2022). Natural sweeteners which are not calorific in addition to non-cariogenic effects on human health in recent years have been greatly developed. These sweeteners are mostly drawn from the diterpenoid glycosides family which is known as steviol glycosides and could be elicited from Stevia (*S. rebaudiana*) leaves (Soejarto, 2001). Stevia, as a low-calorie natural sweetener, is an herb plant that has been cultivated as a crop in many countries, such as Canada, India, Japan, Tanzania, Korea, China, the USA, Paraguay, Mexico, Indonesia, Russia, Brazil, and Argentina (Ramesh *et al*., 2006). This plant belongs to the Asteraceae family and has a 2n = 22 genomic value. Sweet leaves of stevia are applicable to make natural sweet taste in nutrition through its steviol glycosides content such as stevioside and Rebaudioside A, secondary metabolites of the *S. rebaudiana* (Makapugay *et al*., 1984).

In general, stevia leaves have many secondary metabolites that have a broad structure, and these secondary metabolites generally play an essential role

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in plant metabolism. Steviols, glycosides, and ribodiosides are among the secondary metabolites in stevia that increase the natural sweeteners in stevia through the terpenoid pathway. In general, the genetic path of these metabolites starts in the chloroplast and finally ends with the synthesis of ribodiozide. Recent research has shown that glycosides are molecules containing glucose residues, and glycosides are connected with steviols through protein channels. These protein channels finally activate molecular signals and receptors and cause a sweet taste in the plant. As we know, steviol is not digested by the digestive system and enters the bloodstream directly, where it is finally metabolized by the liver and excreted through urine. This feature has made stevia a useful sweetener with zero calories (Peteliuk *et al*., 2021; Watanabe *et al*., 2023).

As mentioned, the consumption of stevia can be useful for diabetic patients. However, recent research has shown that steviols may contain mutagenic compounds, and their excessive consumption can lead to gene mutations. Of course, this research has shown that taking the right dose of these compounds has no concern for human health. However, it should be noted that excessive consumption of any natural or unnatural substance can harm the body (Carakostas *et al*., 2008). nowadays, due to the medicinal properties of stevia, this plant is widely used in the food and pharmaceutical industries (Simoni *et al*., 2024; Drewnowski *et al*., 2019). Sugar control is one of the important features of this plant, and it is very effective, especially for diabetic patients. Ice cream and carbonated soft drink factories use this plant as a sweetener. Also, replacing this plant with sugar in the biscuit formulation reduces the peroxide value of the extracted fat. Furthermore, due to its antioxidant properties, stevia creates a sweet and pleasant taste in producing quality biscuits. Another use of stevia is in baking. Stevia increases the shelf life and improves the bakery's quality of bread. One of the main sweetening factors of this plant, which is widely used in the food industry, is diterpene glycosides, which are used in food additives (Ruiz-Ruiz *et al*., 2015; Pawar *et al*., 2013). Since the sweeteners in stevia are not fermented and are stable at a temperature of 200 degrees Celsius and in acidic conditions, this enables the use of stevia in a wide range of food products (Lemus-Mondaca *et al*., 2012). Today, the European Union has approved the use of stevia in 30 types of food (Ismail *et al*., 2020). Considering that stevia's sweetening substance called glycoside is very important, increasing the efficiency of this substance in the plant and especially in the leaf is a priority. Nowadays, the propagation of stevia is done by increasing the sweeteners in the leaves, and the effect of many substances on this plant has been tested. The results showed that iron and zinc treatments can be effective in addition to the direct effect on the physiological properties of the plant on the genes effective in the production of glycosyl and other sweetening compounds of the plant. In another study, it was found that iron and zinc treatments can increase the production of steviol and glycosides in plants by stimulating cells, inducing a defense response, and producing secondary metabolism (Baroni-Nezhad *et al*., 2021; Javed *et al*., 2017; Castro-González *et al*., 2019; Rai and Han, 2022). Since the seeds of the stevia plant are very small and their germination is very difficult, the use of genetic engineering techniques, especially tissue culture, has greatly helped the cultivation of this medicinal plant. Also, because the leaves of this plant contain valuable compounds, a lot of research has been done to improve these sweeteners and secondary metabolites as much as possible (Zhang *et al*., 2019; Yang *et al*., 2014). In this research, in addition to measuring the biological properties of the plant, we tried to examine the genes effective in the production of steviol. For this reason, in this research, we investigated the effects of different metal oxides on the expression (*CPPS*, *HDS*, and *GGDPS*) of 3 key genes in this pathway.

2. Materials and methods

In this research, the seeds were first obtained from Shahr Raz Agriculture Company. Then the rooted seeds were transferred from the Petri dish to the pot, and sampling was done at the 6-leaf stage. The experiment was conducted with 64 pots in a completely randomized design with three replications. In this study, we tried to use genes that are directly effective in the expression of the active substance Steviol Glycosyl and especially in the production of Rebaudioside A. For this reason, CPPS, HDS, and GGDPS genes were selected for this study based on previous research. As we know, metal oxides are considered as plant growth regulators in plant studies. As a result, the treatment of Cro_3, Pbo, Fe_2O_3, Ag_2O, Bao, and Tio_2 with concentrations of 50, 100 and 200 microliters was done as foliar spraying on the leaves to finally analyze the results. After applying the treatments, leaf samples were taken from the plant. RNA extraction was done with an RNA extraction kit (Dena Zist, Tehran, Iran). After performing NanoDrop to check the quality of the extracted RNA, DNase treatment and cDNA synthesis were performed using the Fermentas kit. Real-time PCR primers were designed using Allele ID software, and then two internal control primers were used to normalize the data (Yang *et al*., 2014; Nicot *et al*., 2005; Livak and Schmittgen, 2001; Larionov *et al*., 2005). Finally, the real-time PCR reaction was performed using the Japanese Takara kit. The list of primers used in the reaction is reported in Table 1. As we know, Ct data is based on a logarithm based on 2, so -ΔΔCt is the fold change in logarithmic form on base 2. Now, in order to make -ΔΔCt linear, 2 must be raised to the power of -ΔΔCt. The resulting values will represent the fold change for each sample. The statistical analysis of this research was performed by SAS software with a probability level of 5%. Samples were prepared to check particle dispersion using an ultrasonic device (Bandlin, Germany). Then hydrodynamic particles and zeta potential were measured in deionized water with a final concentration of 50 micrograms. In the end, the physiological traits of chlorophyll a, b, total chlorophyll (Arnon, 1949; Lichtenthaler *et al*., 1987), carbohydrates (Schlegel, 1956), protein (Bradford, 1976), and proline content (Bates *et al*., 1973) were measured.

Table 1. Sequences of primers used for real-time PCR amplification and the resulting product size.

Primer	Sequence	Amplicon length (bp)	TM
CPPSF	CTACACGGCTTCGCTTTG	113	53.1
CPPSR	GTCACATCTACTCCATCTTGC	113	53.4
GGDPSF	CGATTGGTTTGTTGTTTCAG	169	50.8
GGDPSR	GCTTCCTTGTTTAATTTCTCC	169	50.4
HDSF	TTTCTTGGCTCCGTATCG	178	50.9
HDSR	TGAGGCTACATCTGAATAGG	178	50.6
Elongation Factor-F (internal controls)	GATGCTTCCGACTAAACCTATGG	113	56.6
Elongation Factor-R (internal controls)	CACTCTTGATAACACCGACTGC	113	56.9

3. Results and discussion

3.1. Properties of gelatin metal oxide dispersion

The physical properties of gelation/metal oxide, including conductivity, pH, zeta potential, and particle size, are summarized in Table 2. Gelatin had a similar PH to gelatin/metal oxide. Thus, metal oxide caused a non-significant change in the pH of the gelatin dispersion. Metal oxides can modify gelatin dispersion conductivity, while gelatin/ Ag_2O had the lowest and gelation/ Tio_2 had the highest conductivity. Metal oxide can increase gelatin's zeta potential, while gelatin/ Cro_3 has the highest zeta potential. Metal oxide can decrease gelatin particle size, while gelatin/ Cro_3 has the lowest particle size. Previous studies have reported changes (increase or decrease) in the physical properties of gelation dispersion as a result of metal oxide addition.

The conductivity of the dispersion generally relates to the number of electrical charges (cations and anions ions) in an electrical field. The presence of hydroxy /carboxyl/amino groups results in an electrical charge in the gelatin, to which the high conductivity of pure gelatin could be attributed. At the same time, the alteration in the conductivity of the gelatin dispersion caused by the addition of metal oxide could be attributed to the differential interaction of metal oxide particles with the gelatin chain. An increase in the metal oxide significantly decreased the zeta potential of the gelatin. The decrease in the zeta potential on the surface of the gelatin dispersion could result from the interaction of gelatin and metal oxide. The interaction of metal oxide (positive charge) and gelatin (negative charge) decreases the negative charges on the surface of the particles.

An increase in metal oxide significantly decreased the particle size of the gelatin dispersion. The addition of metal oxide to the gelatin decreased the mean particle size. In gelatin dispersion, interaction between gelatin chains via hydrogen bonds and van der Waals interactions makes a particle with an average particle size of 155 nm. Gelatin with a negative charge can interact with metal oxide particles. Accordingly, metal oxide had differential effects on the zeta potential and particle sizes of gelatin that may be attributed to its different chemical structure.

Table 2. Conductivity, pH, zeta-potential, particle size and viscosity of gelatin enriched by different metal oxides.

	pH	Conductivity (µS/cm)	Zeta-potential (-mV)	Particle size (nm)
Gelatin	6.17±0.93	312±10.2	15±1.34	155±11.4
Gelatin+Ag_2O	5.88±0.66	289±9.8	18±1.78	137±12
Gelatin+BaO	6.42±0.75	315±11.4	17±2.3	144±12.1
Gelatin+CrO_3	6.04±1.01	298±11.1	28±2.56	105±8.9
Gelatin+Fe_2O_3	6.24±0.23	304±7.4	25±1.33	118±13.3
Gelatin+PbO	6.26±0.45	295±7.9	20±2.34	124±10.1
Gelatin+TiO_2	6.29±0.77	323±12	23±2.9	122±8.9

3.2. Gene expression analysis

Three key genes involved in the production of sweetener nutrients in stevia plants, consisting of *CPPS*, *HDS*, and *GGDPS* were assessed to consider their expressional patterns. Fig. 1-3 respectively represent the expressional pattern for *CPPS*, *HDS*, and *GGDPS* under different metal oxide treatments and their different concentrations. Regarding the *CPPS* gene under the Tio_2 with 100 µmol/ml concentration treatment, the highest expression value with no statistical significance in comparison to Fe_2O_3 was obtained. Furthermore, Pbo under both 50 and 200 µmol/ml concentration, Ag_2O under both 100 µmol/ml concentration, Tio2 under 200 µmol/ml concentration, Fe_2O_3 under 200 µmol/ml concentration, and also Cro_3 under 200 µmol/ml concentration, no statistical significant was observed (Fig. 1). On the contrary, the rest of the treatments showed the lowest expression values without statistically significant differences, which among Ag_2O under 200 µmol/ml concentration and control were the lowest ones. On the whole, Tio2 and Fe_2O_3 could be bold as the most effective treatments for up-regulating this gene under high concentrations (100 and 200 µmol/ml concentration).

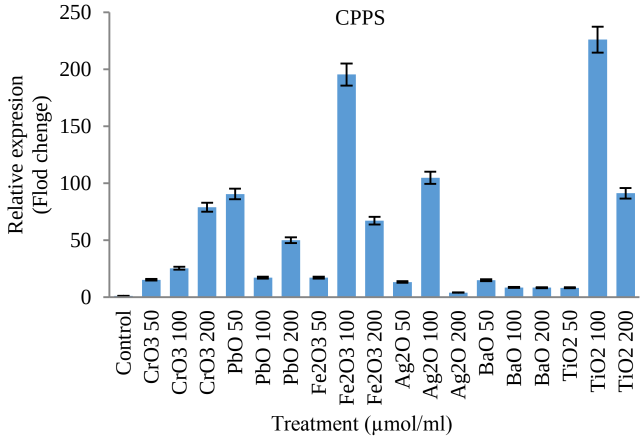

Figure 1. Expression pattern in response to different metal oxides treatments regarding *CPPS* gene.

HDS gene expression showed the highest value in response to 100 µmol/ml concentration of Fe_2O_3. Tio_2 led to a little lower expression pattern for the *HDS* gene than Fe_2O_3, whereas it was not significantly different from the expression value resulting from that in Fe_2O_3. On the other hand, Tio_2 itself showed no significant difference with Cro_3 under 200 µmol/ml concentration. Consequently, Ag_2O and Tio_2 respectively fewer than 100 and 200 µmol/ml concentration with no significant difference, showed lower expression values than those mentioned before. After these treatments, Pbo and Fe_2O_3 both at a concentration of 200 µmol/ml resulted in no significant difference related to this gene expression. All the other remaining treatments pointed out no difference from one another regarding HDS expression value. However, Control and Cro_3 under 50 µmol/ml concentration in addition to Ag_2O under 200 µmol/ml concentration were the lowest ones (Fig. 2).

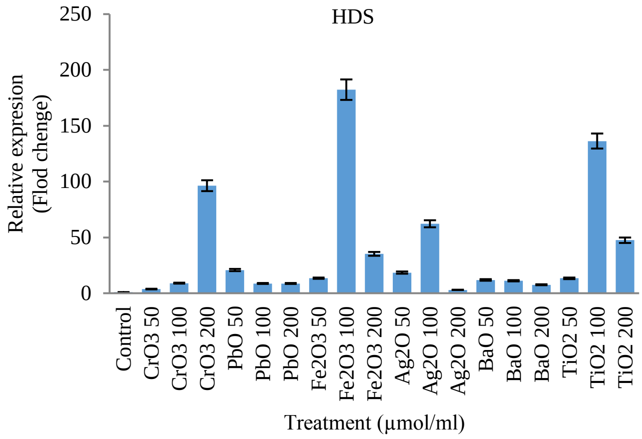

Figure 2. Expression pattern in response to different metal oxides treatments regarding *HDS* gene.

Results of comparison of applied treatments regarding *GGDPS* expression pattern pointed out approximately similar results to *HDS* in which Fe_2O_3 at a concentration of 100 µmol/ml led to the highest expression values with no significant difference from Tio_2 at a concentration of 100 µmol/ml. Also, Pbo under the concentration of 50 µmol/ml was not significantly different from the above-mentioned treatments. Control, in line with Ag_2O and Bao both at a concentration of 50 µmol/ml add up with Cro_3, Pbo, and Bao, all of them at 100 µmol/ml concentration, which resulted in the lowest expression values of the *GGDPS* gene. About all treatments under 200 µmol/ml concentration have approximated high expression values (Fig. 3).

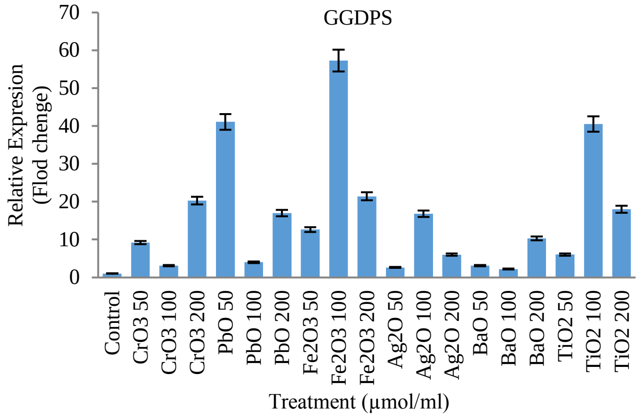

Figure 3. Expression pattern in response to different metal oxides treatments regarding *GGDPS* gene.

In general, it can be concluded that the increased concentration of metal oxides, especially Fe_2O_3 and TiO_2, is escalating gene expression involved in the biosynthesis of sweeteners extracted from stevia leaves. Also, related to all treatments, the higher the concentration was, the higher the gene expression became. Among all metal oxide treatments, Pbo and Bao resulted in low gene expression for genes *CPPS*, *HDS*, and *GGDPS*. On the other hand, control showed the lowest expression regarding all three surveyed genes, indicating that using metal oxides can achieve higher production of sweeteners in stevia plants.

CPPS, *HDS*, and *GGDPS* are a widely divergent group of enzymes that transfer a sugar residue from an activated donor to an acceptor molecule (Ross *et al.*, 2001). In Stevia, *CPPS*, *HDS*, and *GGDPS* were proposed to be involved in the production of rebaudioside A, which is unique in the plant world because of its intense sweetness and high concentration in the leaf tissue. Chen et al. (2014) found 161 unigenes that were predicted to encode UDP-glycosyltransferases, including *CPPS*, *HDS*, and *GGDPS*, which have been reported to be involved in the rebaudioside A biosynthetic pathway.

3.3. Biochemical study

Fig. 4 shows the content of different pigments measured in stevia plants under different treatment conditions. Chlorophyll and total chlorophyll increased significantly in response to the increase in the concentration of metal oxides. The content of chlorophyll b showed a low difference between plants under 50 and 100 µmol/ml but the 200 µmol/ml showed a higher difference in comparison to the aforementioned concentrations. The results of carotenoid content showed a similar pattern to chlorophyll b with a slight difference related to 200 µmol/ml. CrO_3 and PbO showed higher content of chlorophyll and total chlorophyll than other treatments under 50 µmol/ml, while they showed lower content under two other concentrations. Ag_2O and TiO_2 showed the highest content of chlorophyll a and total chlorophyll under 100 and 200 µmol/ml, respectively (Fig. 4). Chlorophyll b and carotenoid showed no significant difference related to metal-oxidants under 50 and 100 µmol/ml between TiO_2 showed the highest content under these two types of pigments. Under stress conditions, usually, the color of leaves goes lighter and the overall content of pigments goes lower. Despite decreasing the content of pigments, the overall area of the leaves gets smaller. Hence, it is probable that using these metal-oxidants has negative effects similar to stress conditions, so the pigment content of leaves gets higher at last. Also, it is proved in the literature that carotenoids can act as non-enzymatic antioxidants, which is an illustration of their increase under higher concentrations of metal oxides.

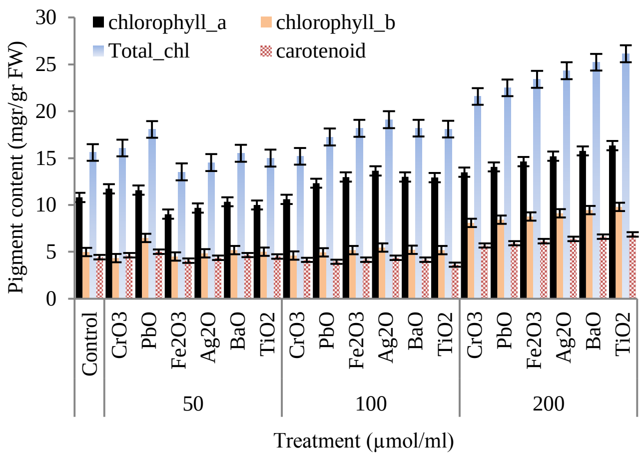

Figure 4. Pigments content measured in Cro_3, Pbo, Fe_2O_3, Ag_2O, Bao, and Tio_2 treatments.

The content of free proline is generally enhanced along with the increase in the concentration of metal oxides (Fig. 5). Under 50 µmol/ml concentration, CrO_3 revealed the highest free proline content, while the lowest content was recognized in BaO with no significant difference to TiO2 and Ag_2O. Whereas CrO_3 had a maximum content of free proline under 50 µmol/ml, it showed the lowest content under 100 µmol/ml in line with PbO. Under 100 µmol/ml TiO_2 obtained the highest proline content without a significant difference from BaO. Ag_2O, despite its low content between 50 and 100 µmol/ml, showed a maximum content of free proline under 200 µmol/ml. Under this condition, Fe_2O_3 and Ag_2O do not reveal any significant differences, while TiO_2 showed the lowest content of free proline, a highly significant difference between the others. It is well known that osmotic regulators such as proline, potassium, and soluble sugar are small molecules relevant for evaluating osmotic adjustment ability and drought resistance in plants (Chen and Gallie, 2004). The content of free proline is another reason for the probable stressful conditions that metal oxides may induce.

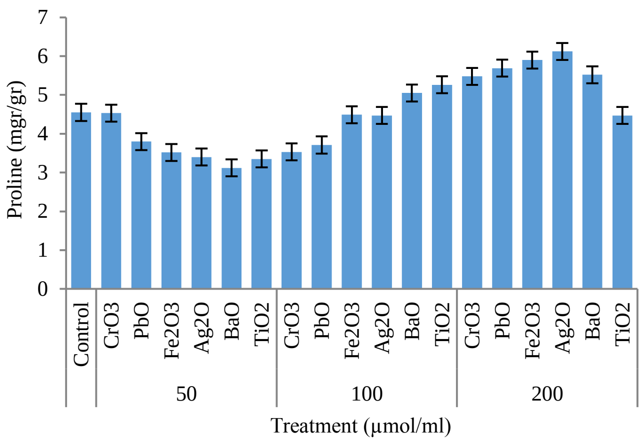

Figure 5. Proline content measured in Cro_3, Pbo, Fe_2O_3, Ag_2O, Bao, and Tio_2 treatments.

Also, the amount of soluble carbohydrates is enhanced with the increase in the concentration of metal oxides (Fig. 6). Under 50 µmol/ml and 100 µmol/ml concentration, CrO_3 revealed the lowest soluble carbohydrate content, while the highest content was recognized in Ag_2O and BaO. Under 200 µmol/ml Ag_2O obtained the highest carbohydrate content while TiO_2 showed the lowest carbohydrate content.

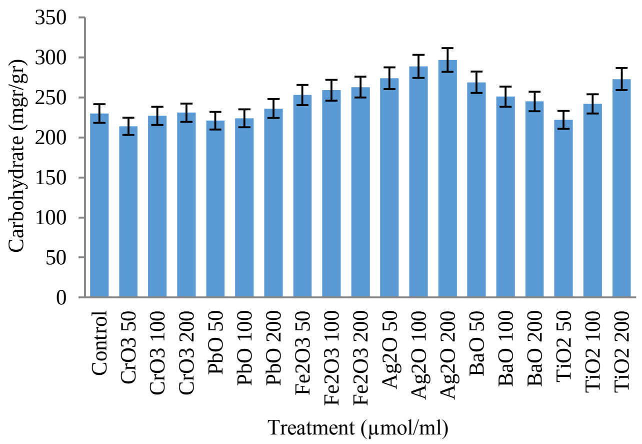

Figure 6. Carbohydrate content measured in Cro_3, Pbo, Fe_2O_3, Ag_2O, Bao, and Tio_2 treatments.

The content of protein is generally enhanced along with the increase in the concentration of metal oxides (Fig. 7). Less than 50 µmol/ml concentration, CrO_3 revealed the lowest protein content, while the highest content was recognized in Ag_2O with no significant difference to BaO. Under 100 µmol/ml Ag_2O obtained the highest protein content without a significant difference to Fe_2O_3.

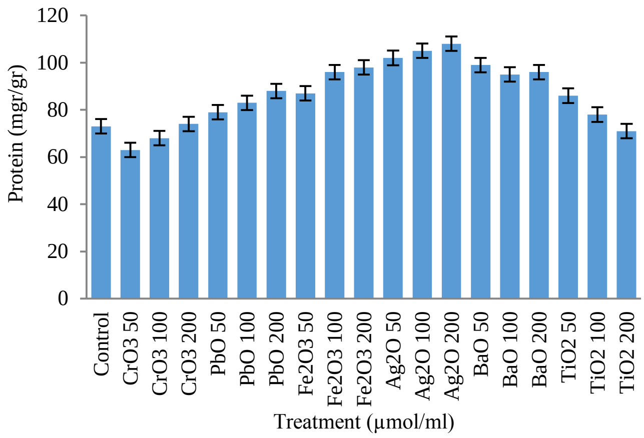

Figure 7. Protein content measured in in Cro_3, Pbo, Fe_2O_3, Ag_2O, Bao, and Tio_2 treatments.

Under 200 µmol/ml Ag_2O and TiO_2 obtained the highest protein content while CrO_3 and pbo showed the lowest protein content. According to current spraying and treatment of metal oxide plants, in the absence of a similar situation with drought or salinity, on the other hand, as we know, the accumulation of organic compounds such as carbohydrates and amino acids in the cytoplasm plays an important role in the regulation of osmotic plants. It can be seen that the increase of organic compounds in plant defense response is to deal with this stress.

The Mettler-Toledo instrument was used to measure the pH and conductivity of the dispersion. The zata-potential and hydrodynamic particle sizes were determined using a Brookhaven Instruments Corporation 90 Plus zeta-sizer as reported in materials and methods. The apparent viscosities (mPa.s) of the dispersions were quantified through the use of Brookfield Viscometer (spindle 02) at spindle rotational speed of 10 s^{-1}. The values are expressed as means ± standard deviation for three independent experiments. Mean values with different letters within a column are significantly different by Duncan test at (p<0.05). Gelatin had a similar pH to gelatin/metal oxide. Metal oxide can modify gelatin conductivity while gelatin/Ag_2O had the lowest conductivity. Metal oxide can alter gelatin zeta-potential and particle size while gelatin/CrO_3 had the lowest particle size and the highest zeta-potential. Metal oxide can alter gelatin viscosity while gelatin/CrO_3 had the highest viscosity.

4. Conclusion

This research and other similar researches show that the use of metal oxides and micronutrients will have important effects on the production of secondary metabolites and antioxidants. As we have seen in this research, determining the appropriate dose has a great impact on increasing the effective ingredients of the plant, and failure to comply with this can affect and disrupt other compounds. Finally, the use of these compounds will have a promising perspective in the future, which will lead to positive approaches in effective compounds and secondary metabolites in medicinal plants, especially stevia.

Conflict of interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No human or animals were used in the present research.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

All authors had an equal role in study design, work, statistical analysis and manuscript writing.

Informed consent

The authors declare not to use any patients in this research.

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